REGULATION OF REGENERATIVE ABILITY OF SOMATIC EMBRYOS OF SWEETPOTATO (IPOMOEA BATATAS (L.) LAM.): MORPHOLOGICAL AND MOLECULAR APPROACHES

By

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TABLE OF CONTENTS

PAGE

ACKNOWLEDGMENTS ii

ABSTRACT v

CHAPTERS

1 INTRODUCTION 1

2 LITERATURE REVIEW 5

Introduction 5
Historical Background 7
Auxin and Embryogenesis 11
Auxin and Regulation of Gene Expression 15
Auxin and Cell Cycle 22
Auxin Binding Proteins 25
Concluding Remarks 27

3 HISTOLOGICAL COMPARISONS OF SHOOT FORMING AND NON SHOOT FORMING SOMATIC EMBRYOS OF SWEETPOTATO (*Ipomoea batatas* (L.) Lam.) 26

Introduction 29
Material and Methods 31
Results 34
Discussion 54
Summary 58

4 COMPUTER VISION ANALYSIS OF SOMATIC EMBRYOS FOR ASSESSING THEIR CONVERSION TO PLANTLETS 60

Introduction 60
Material and Methods 63
Results 66
Discussion 85
Summary 95

5 DIFFERENTIAL AUXIN AND SUGAR-RESPONSIVE EXPRESSION IN EMBRYOGENIC CALLUS CULTURES OF SWEETPOTATO (*Ipomoea batatas* (L.) Lam.) 97

Introduction 97
Material and Methods 100
Results 103
Discussion 134
Summary 151

6 SUMMARY AND CONCLUSION 152

LITERATURE CITED 155

APPENDIX 173

BIBLIOGRAPHY 174

Abstract of Dissertation Presented to the Graduate School
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REGULATION OF REGENERATIVE CAPACITY OF SOMATIC EMBRYOS OF SWEETPOTATO (IPOMOEA BATATAS (L.) LAM.): MORPHOLOGICAL AND MOLECULAR APPROACHES

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Effective use of somatic embryos for transformation or synthetic seed technology requires production of homogenous embryo populations that readily form plantlets. Conversion ability of sweetpotato somatic embryos were examined both at the morphological and molecular level.

From the 80% embryos that formed roots, only 44% produced whole plants. Five major morphological variants among torpedo and cotyledonary stage embryos were observed. These included 1) Perfect Type, 2) Near Perfect Type, 3) Limited/No Meristematic Activity Type, 4) Disrupted Internal Anatomy Type, 5) Proliferating Type. Perfect and Near Perfect Types of somatic embryos were found to be competent, while Limited/No Meristematic activity, Disrupted Internal Anatomy and Proliferating Types were found to be noncompetent with respect to their ability to convert into plantlets. Lack of organized shoot development in sweetpotato somatic embryos was associated with the following abnormalities: 1) lack of an organized apical meristem 2) sparsity of dividing cells in the apical region 3) flattened apical meristem 4) multiple meristemoids and/or diffuse meristematic activity throughout the embryo. The morphological fate of most torpedo and cotyledonary embryo variants was mapped.

Computer vision and Canonical or Fisher Discriminant Function (CDA) analysis correctly classified competent embryos about 45% of the time based on size, 55% based on shape and 57% based on color. Noncompetent embryos were correctly classified 70% of the time based on size, 57% based on shape and 73% based on color. Of the two classification analyses used, CDA was found superior.

Age decreased the capacity of callus lines to generate developmentally competent embryos, so potential control aspects of gene expression were compared between embryogenic cultures designated new (K1194 and K195) and old (K592 and M892). Analyses included conversion studies and Northern blot hybridization with heterologous auxin-responsive gene probes (pJCW1 and pJCW2, Ainley et al. 1988) and sugar-responsive gene probes (*Ivr2* and *Sh1*, Koch et al. 1992, 1995). Embryos from new lines formed more shoots, roots and whole plants compared to embryos from old lines. Newer lines had higher auxin-responsive gene expression, while sugar-responsive gene expression showed no differences between lines. These results indicated that decreased developmental competence in callus lines may be related to desensitization of auxin-responsiveness. Improved regeneration efficiency is associated with developmental competence of the somatic embryos.

CHAPTER 1
INTRODUCTION

Root and tuber crops, including sweetpotato (*Ipomoea batatas* (L.) Lam), feed millions of people in developing countries (Gregory, 1992). Roots and greens of sweetpotato can provide nutrition for more people per unit hectare than any other crop (Woolfe, 1992). Also, sweetpotato is a nutritious source of food containing substantial amount of proteins, riboflavin, β-carotene, ascorbic acid and tocopherol (Woolfe, 1992). In addition, sweetpotato is also used as a fast growing ground cover to prevent soil erosion, a relay crop system that can tolerate severe weather condition, animal feed (Gregory, 1992) and an alternate energy source that can be used for the fuel production (Smith and Frank, 1984).

Sweetpotato is an important crop in many areas of the world (Bouwkamp, 1985) and the major sweetpotato producers are Asia, followed by Africa and South America (Pierce, 1987). Sweetpotato may have a larger impact in the future as a vital food source serving the poorest and fastest growing populations of the world. Improved production of sweetpotato through the introduction of value-added traits such as virus resistance, herbicide resistance and improved nutritional quality have been a main focus of recent studies (Gama et al., 1992). The introduction of such value-added traits through conventional breeding is almost impossible because of the complexity of the sweetpotato genome. The introduction of foreign genes using recombinant technology and tissue culture techniques have been reported in sweetpotato (Gama et al., 1992 Prakash and Varadarajan 1992, Nishiguchi et al 1992) and in many other plant species (Klee and Estelle 1991) Gama et al (1992) successfully produced transgenic plants from somatic embryos obtained from embryogenic calli transformed with *Agrobacterium tumefaciens* The process required a good somatic embryogenic system with competent embryo production

Use of sweetpotato starches for methane production requires large scale propagation of sweetpotato Vegetative propagation for this purpose would be very costly compared to the returns obtained from the methane produced (Cantliffe 1987) In sweetpotato somatic embryogenesis is a very effective method of propagation because of its clonal nature and capacity to mass produce somatic embryos which would eventually giving rise to plants (Chee et al., 1992) The torpedo and cotyledonary stage embryos have the potential to be used as functional seeds referred as "synthetic seeds" (Gray and Purohit 1991) for large scale production of sweetpotato

Use of somatic embryos for transformation or synthetic seed technology requires a fairly homogenous population of competent embryos with uniform and high rates of conversion However this is very difficult to achieve because the population of somatic embryos in culture typically are highly heterogeneous A variety of embryonic types develop through somatic embryogenesis and show great variability in developmental patterns and conversion to plantlets (Ammirato 1985 Chee and Cantliffe 1988) This challenges the biological researcher to identify the competent embryos that would eventually give rise to plants (Harrell et al 1993 1994)

Use of computer vision analysis to qualify and quantify research in somatic embryogenesis has been demonstrated previously (Grand d'Esnon et al 1989 and Cazzulino et al 1990) Computer vision analyses can serve as a tool to select competent somatic embryos (Harrell et al 1994) Application of computer vision technology along with histological analysis can identify morphogenic variants in somatic embryos with various developmental patterns and could identify competent somatic embryo morphologies.

Long term production of competent embryos is necessary for practical application of somatic embryogenesis However in callus lines of sweetpotato morphogenetic potential for plantlet conversion decreases with age One hypothesis for this change is altered auxin and/or sugar responsiveness with callus age Auxins influence not only those developmental sequences that precede embryo formation but also subsequent morphogenesis events in embryo development (Schiavone and Cooke 1987 Michalczuk et al, 1992 Zimmermann 1993) in somatic embryogenesis In addition sucrose concentration in the media also influences the embryo initiation and differentiation process (Meijer and Brown 1987) in somatic embryogenesis

Auxin induced growth and development involves changes in gene expression (Theologis 1986) Several cDNAs corresponding to auxin induced RNA's have been isolated These include GH1 GH2 GH3 GH4 SAUR (small auxin-up RNA's) in soybean (Hagen et al 1984 Hagen et al 1988 McClure and Guilfoyle 1987) pJCW1 and pJCW2 (Walker and Key 1982) These cDNA clones can be used as markers for auxin-induction (Hagen et al. 1984 Hagen et al. 1988) Sucrose utilization for cellular mechanisms in plants is effected through a group of genes which are modulated in response to changes in sugar levels (Koch et al 1995) These genes include sucrose synthase and invertase which are regulated so as to adjust the capacity for sucrose utilization (Koch et al 1995)

The objectives at this research with sweetpotato somatic embryos are three fold

1) Elucidate the developmental pathways of somatic embryos by comparing the external morphology (captured by machine vision) with thes internal anatomy and thereby identify the competent and noncompetent morphogenic variants among torpedo and cotyledonary stages

2) Identify developmentally competent and noncompetent classes of somatic embryos using size shape and color related measurements extracted by applying a machine vision system (Harrell et al 1994) and relate these classes to the information obtained from the first objective to more positively distinguish between competent and noncompetent somatic embryo variants by external embryo morphology

3) Identify altered auxin- and sugar-responsive gene expression in embryogenic callus of sweetpotato with competent embryo production This hypothesis was tested by comparing levels of mRNA hybridizing to heterologous auxin-responsive and sugar-responsive gene probes in different aged calli having different capacities to generate developmentally competent embryos In addition different embryo types obtained through the first objective were tested to determine the alterations in auxin responsive expression with morphological competence in these embryos

CHAPTER 2
LITERATURE REVIEW

Introduction

In many flowering plants in addition to sexual reproduction by which new plants are formed from the zygote asexual reproduction of new plants arising from fragments of organs tissues or somatic cells can lead to the production of new plants Somatic embryogenesis can be defined as the developmental process by which haploid or diploid somatic cells develop through different embryogenic stages to give rise to a whole plant without the fusion of gametes (Williams and Maheswaran 1986)

Sharp et al (1980) distinguished two developmental patterns namely direct and indirect embryogenesis Direct embryogenesis occurs directly from tissues without callus proliferation whereas indirect embryogenesis requires callus maintenance and proliferation before embryo formation.

Somatic embryogenesis apart from being an important technique for plant propagation serves as a tool for basic research in plant embryo development and other aspects of plant physiology (Yeung 1995) This process has received much attention as it provides an ideal system to produce transgenic plants and synthetic seeds

Mechanization of some or all of the stages of embryo production and plant regeneration is required for commercialization of somatic embryos as a synthetic seed system (Smith 1995) Mass production of embryos can be accomplished by growing the embryos in controlled bioreactor systems, automated recognition and selection of mature somatic embryos encapsulation and delivery of embryos to the field This commercialization of the synthetic seed system has been hindered by the heterogeneity of embryos both in terms of quality and maturity (Harrell et al 1993)

Production of a synchronous population of quality mature embryos with high conversion ability is a prerequisite for synthetic seed system (Cantliffe et al 1993) First optimization of the culture conditions to produce synchronous singulated quality embryos is required Second, machine vision with suitable algorithms to classify somatic embryos based on a comparison of visual morphological observation with subsequent embryo conversion is necessary

Auxin is the most important factor involved in the induction and development of embryogenesis (Kawahara and Komamine 1995) In addition to a critical role in generating the embryogenesis auxins typically 2 4-D inhibit further embryo development. Moreover synthetic auxin such as 2 4-D has been suggested to cause genetic and epigenetic changes in embryogenic cultures that have an effect on continued embryogenic potential (Caligari and Shohet 1993)

In the review that follows some of the advances made at both the physiological and molecular level for understanding the relationship between auxin and somatic embryogenesis is explored

Historical Background

Auxin

Auxin was the first hormone discovered in plants (Moore, 1989). As early as 1880, Charles Darwin postulated that some transmitted influence from the shoot tip to the lower part of the plant was responsible for phototropism. Of the many investigators who extended Darwin's experiments, it was Went (1928) who finally discovered that the transmitted substance was auxin, through his study with *Avena* coleoptiles. The term auxin was originally proposed by Kogl and Haagen-Smit (1931) and was derived from a Greek word "auxein" which means to grow. Following its discovery, IAA was isolated from yeast, fungi, bacteria, algae and higher plants and it is now accepted that IAA is the only indole native auxin of higher plants (Moore, 1989). A nonindolic auxin, namely phenylacetic acid (PAA), also occurs naturally in some plants (Moore, 1989).

Many synthetic compounds that have physiological properties similar to IAA were identified and these compounds were categorized into five major groups, namely indole acids, naphthalene acids, chlorophenoxy acids, benzoic acids and picolinic acid derivatives (Moore, 1989). These synthetic compounds are also referred to as auxins (Moore, 1989). Most commonly used synthetic auxins in tissue culture include naphthalene acetic acid (NAA) of the naphthalene acid group and 2,4-D (2,4-dichlorophenoxyacetic acid) of the chlorophenoxy acid group.

The biosynthetic pathway of IAA is still at controversy. Owing to its 3-substituted indole structure similar to tryptophan, as early as 1930 it was theorized that IAA was derived from tryptophan (Normanly et al., 1995). Studies with labeled and unlabelled tryptophan incorporation into IAA in the 1940s and 1950s concluded that IAA was derived from tryptophan (Normanly, 1995). However, quantitative in vivo labeling studies with [^{15}N] in *Lemna* (Baldi et al., 1991) and *Trp* mutants of Arabidopsis and Maize (Normanly et al., 1993; Wright et al., 1991) questioned whether Trp is the precursor to IAA. In *Lemna*, feeding of [^{15}N] incorporated 98% of the label the Trp pool, but very little IAA was labeled (Baldi et al., 1991). *Orange pericarp*, a maize mutant defective in the Trp synthase gene (Wright et al., 1991) and *trp2* mutant of *Arabidopsis* (Normanly et al., 1993) defective in one of the two Trp synthase β genes were found to accumulate IAA. In vivo labeling studies with [^{15}N] anthranilate in these studies revealed that more [^{15}N] was incorporated into IAA than into Trp. However, in vivo labeling studies of bean seedlings showed that all of the IAA was derived from Trp (Bialek et al., 1992).

Michalczuk et al. (1992b) showed the existence of multiple developmentally regulated IAA biosynthetic pathways during carrot somatic embryogenesis. They reported that carrot cells proliferating in the presence of 2,4-D, synthesized IAA from Trp, but in cells undergoing embryogenesis with 2,4-D removal, the IAA was produced via a Trp-independent pathway.

Levels of free IAA in plants are generally regulated by conjugation (Sztein et al., 1995), synthesis and degradation of IAA (Moore, 1989). IAA conjugates are predominantly of two forms, namely esters (with glucose and *myo*-inositol) and amide forms (with aminoacids) (Cohen and Bandurski, 1982). Sztein et al. (1995) reported that major evolutionary events in land plants are accompanied by regulation of IAA through their study on the complexity of conjugation patterns in one charophyte and 23 land plants ranging from liverworts to angiosperms. They identified three conjugation patterns involving no IAA conjugation in charophyte and liverworts, conjugation of IAA into a few amide and ester conjugates in mosses and hornwort, conjugation of IAA into a variety of amide and ester conjugates in tracheophytes and vascular plants.

Auxin and its synthetic analogs added to tissue culture media are also conjugated and metabolized (Kleczkowski and Schell 1995). IAA-Glc synthase gene (iaglu) involved in IAA-metabolism has been cloned recently in maize (Szerszen et al. 1994). Overexpression of this gene in tobacco showed reduced apical dominance and weak gravitropism (Normanly et al. 1995).

Enzymatic catabolism of IAA occurs via two major pathways (Moore 1989). The first, namely IAA oxidase (peroxidase), catalyzed the decarboxylation of IAA leading to a dominant product, namely to 3-methylene-oxindole. The second route is a nondecarboxylation route to oxindole-3-aceticacid and di-oxindole acetic acid (Moore 1989). The first pathway was challenged in that IAA oxidase in plants is insignificant and over or under expression of peroxidase levels in transgenic plants has no effect on IAA levels (Normanly et al. 1995). However, an enzyme that catalyses the second pathway has been found in similar quantities as that of IAA in maize (Normanly et al. 1995).

Polar auxin transport was recognized as early as 1928 by Went (Moore 1989). Auxin is the only plant hormone which is transported polarly. The movement is predominantly basipetal in the shoot and acropetal in the root (Moore 1989). This directed movement of auxin plays a regulatory role in many growth and developmental processes of plants including activation of the vascular cambium, inhibition of lateral buds and control of abscission (Wilson and Wilson 1993). This polarity of movement has also been demonstrated for synthetic auxins such as IBA, NAA and 2,4-D (Moore 1989).

Somatic Embryogenesis

The recognition of somatic embryogenesis in plants started with the demonstration of totipotency by Haberlandt in 1902 Much of the understanding of somatic embryogenesis came from earlier studies with carrot cultures It was pioneering independent experiments of Steward et al (1958) and Reinert (1958) that produced a proliferating mass of callus and subsequent regeneration into normal plants from cultured carrot cells in a medium containing coconut milk which demonstrated the totipotency of plant cells

Following these studies it was shown that practically any organ or tissue of carrot could proliferate on a defined medium containing only minerals sucrose, thiamine and auxin (Halperin 1966 Halperin and Wetherell 1964) Reinert (1959) Wetherell and Halperin (1963) and Steward et al (1963) demonstrated the ability of somatic cells to closely follow the developmental sequence of zygotic embryos The ability to form a completely organized plant from a single somatic cell in tobacco and carrot was demonstrated by Vasil and Hildebrandt (1965) and Backs-Husemann and Reinert (1970) respectively

During the late 1960s and early 1970s investigators recognized the fundamental differences in the early division sequences of zygotic and somatic embryos of carrot Halperin (1966) found that the embryoid appeared as a globular structure from the outermost cells of disorganized embryogenic clumps called proembryogenic masses or PEMs (Halperin 1970) In the continued presence of auxin development of organized embryo from PEMs was inhibited

Embryo development proceeded with root and shoot polarity (root pole always towards the inside of the clump and the shoot pole toward the outside of the clump) when auxin was removed or when the concentration of auxin fell (as in an old culture) (Halperin 1970) The non-dividing cells of the original aggregate remained attached to the root pole of the embryo as a suspensor-like appendage (Halperin 1966 1970) McWilliams Smith and Street (1974) showed that the first division of an embryogenic initial in a cell aggregate is transverse The longitudinal division of a terminal daughter cell results in the embryo proper while transverse division of the cell closest to the cellular aggregates resulted in a suspensor like structure Although early division sequences of zygotic and somatic embryos are different identical structures result at the end of both developmental programs (Raghavan 1986) In the years that followed somatic embryogenesis has been demonstrated in numerous plant species and families (Tisserat 1979 Ammirato 1983) Now somatic embryogenesis has been reported over 300 plant species (Bajaj 1995)

Auxin and Embryogenesis

Auxins play an important role in many processes of growth and differentiation including cell enlargement cell division vascular differentiation apical dominance and root formation Both exogenous and endogenous auxin are also closely involved during the process of somatic embryogenesis (Michalczuk et al 1992a)

Fujimura and Komamine (1979) suggested that a critical level of endogenous auxin level may be neccessary during the early period of embryogenesis in order to assess the sensitivity of carrot cells to auxin and antiauxins A twofold increase in embryoid formation with 2 4-D and no embryogenesis without 2 4-D in the medium was reported by Fujimura and Komamine (1980) Komamine et al (1992) suggested that the presence of auxin is required for the formation of embryogenic clusters from single cells and to express totipotency of competent cells. Michalczuk et al. (1992a) reported that exogenously applied synthetic auxin namely 2,4-D stimulated the accumulation of endogenous IAA up to 600ng/g fresh weight in carrot suspension cultures. This accumulation of endogenous IAA allowed maintenance of proliferative state of callus and prevented subsequent embryo formation (Michalczuk et al., 1992a).

A number of studies have demonstrated that by removing or decreasing the auxin concentration in the regeneration medium the embryo would develop to maturity (Montague et al., 1981). Similar results were obtained with other auxins such as NAA, IBA or IAA (Kamada and Harada, 1979). A better understanding of the role of auxin in embryo formation came from the studies on habituated callus of Shamouti Orange and γ-irradiation studies (Kochba and Spiegel-Roy, 1977). Kochba and Spiegel-Roy (1977) showed that auxin level was one of the factors that controlled embryogenesis. They showed through γ-irradiation that embryo growth could be stimulated following inactivation of endogenous levels of auxin (Kochba and Spiegel-Roy, 1977). Kuleck and Cohen (1992) reported that upon transfer of carrot cells to a 2,4-D lacking medium, there was a dramatic decrease in auxin-amino acid conjugate. They suggested that this reduction in auxin-aminoacid conjugates may result from the appearance of developmentally regulated IAA-conjugate-hydrolyzing activity that may play an important role in embryogenic induction. They also partially purified and determined the kinetic properties of an IAA-alanine hydrolase.

Recently, Michalczuk et *al.* (1992a) evaluated the changes in concentration of 2,4-D and IAA in developing embryos to assess the role of auxin levels in embryo development. They showed that removal of carrot callus cells to 2,4-D free medium resulted in a decrease in total IAA that was low enough to set up an internal gradient for stages of embryo development Furthermore they showed that addition of auxins namely IAA and 2 4-D at a concentration of 1μM to globular and heart stage of embryos prevented development to next stage namely heart and torpedo stage respectively This developmental arrest was much more pronounced with application of 2 4-D than IAA and no torpedo stage embryos developed when 2 4-D was applied to heart stage embryos (Schiavone and Cooke 1997) These observations suggested that development of embryos successively through each stage required lower IAA levels than the preceding stage (Michalczuk et al 1992a) Exogenous application of auxin to various embryo stages can lead to perturbation of a low internal gradient of IAA level or to embryos that become increasingly sensitive to exogenous auxin as they proceed through later developmental stages (Michalczuk et al 1992a)

A critical role of polar auxin transport in plant embryogenesis has been reported for a long time in the literature Fry and Wangermann (1976) postulated that the initiation of polarized auxin transport in the globular embryos determined the morphological polarity expressed during later stages of embryo development

Wilson and Wilson (1993) reported that the direction of auxin movement in the surrounding parental tissues controls initiation of polarity in zygotic embryos They suggested that in an exoscopic embryo (where shoot apex of embryo develops form the upper of the two cells formed by the first division of the zygote) of lower plants the direction of polar auxin transport is similar as that of the parent plant. This maintains the direction of auxin polar transport consistent from one generation to another On the other hand in an endoscopic embryo (where the shoot apex of the embryo develops form the lower of the two cells formed by the first division of the zygote) of seed plants the polarity is reverted between parent plant and embryos such that direction of auxin transport is inverted in the new generation (Wilson and Wilson, 1993)

When polar auxin transport was blocked at the globular stage of cultured zygotic embryos of Indian mustard (*Brassica juncea*) it led to the formation of fused collar like cotyledons (Liu et al 1993) resembling the pin-1 mutant of Arabidopsis that has reduced auxin transport in its inflorescence (Okada et al 1991) Application of auxin transport inhibitors to heart stage embryo failed to induce such an abnormality This led to the suggestion that polar auxin transport was required for cotyledon initiation for a short period during the globular stage (Liu et al 1993)

The role of polar auxin transport during somatic embryo development was determined through the use of polar auxin transport inhibitors such as TIBA (2 3 5-triiodobenzoic acid and NPA N-(1-naphthyl)phthalamic acid These transport inhibitors effectively block auxin transport by binding to the regulatory site of auxin efflux carrier complex (Schiavone and Cooke 1987 Venis 1985) These inhibitors blocked subsequent development of somatic embryos i e the globular and oblong (intermediate stage between globular and heart) embryos failed to continue their development to the next stage Instead they continued to increase in size without any cotyledon initiation (Schiavone and Cooke 1987) Addition of auxin transport inhibitors to heart stage embryos resulted in a failure to develop torpedo embryos (Schiavone and Cooke 1987 Michalczuk et al 1992a)

Chee and Cantliffe (1989) through the use of TIBA and/or 2 4-D additions showed that polar auxin transport was inhibited by exogenous application of auxin resulting in inhibition of embryogenesis in sweetpotato Similar results were reported with the use of difluoromethylornithine (DFMO) a key enzyme that is involved in putrescine biosynthesis This enzyme counteracts the inhibition of somatic embryogenesis by auxin and auxin transport inhibitors but not the inhibition of somatic embryogenesis by antiauxin that had no effect on polar auxin transport (Robie and Minocha 1989 Nissen and Minocha 1993)

Furthermore microsurgical experiments involving regeneration of shoot and root structures from cut apical and basal ends of a somatic embryo and sensitivity of the regeneration process to exogenous auxin and auxin transport inhibitors supports the importance of polar auxin transport in regulating polarity in somatic embryo development (Schiavone and Racusen 1990 1991)

Auxin and Regulation of Gene Expression

Molecular and genetic analysis of auxin action in recent years were aimed at gaining an understanding of the mechanism of auxin action as well as functional responses to the auxin signal These analyses included isolation and characterization of auxin-regulated genes (Ho and Hagen 1993) study of mutants altered in auxin metabolism and perception (Barbier Brygoo 1995) and alteration of auxin levels through transformation techniques with genes encoding enzymes involved in auxin biosynthesis (Klee and Romano 1994)

A plant hormone such as auxin exerts its regulatory role by inducing growth and developmental processes via gene expression (Theologis 1986 Ho and Hagen 1993) Auxin application to plants or excised plant organ such as soybean hypocotyl (Hagen et al. 1984) or tobacco cell cultures (Van der Zaal et al 1987) leads to the rapid increase of many mRNA s and /or genes

Several cDNAs hybridizing to auxin induced RNA s have been isolated from soybean such as GH1 GH2 GH3 GH4 SAUR (small auxin-up RNA s) (Hagen et al 1984 Hagen et al 1988 McClure and Guilfoyle 1987) and pJCW1 and pJCW2 (Walker and Key 1982) In addition pIAA (Theologis et al 1985) pIAA4/5 pIAA6 (Oeller et al 1993) have been identified in pea and AGR 1 through 7 (Yamamoto 1992 a b) In mung bean SAR1 and SAR2 (Reddy 1990) have been obtained from strawberries pCNT103 pCNT107 pCNT114 (Boot et al. 1993) pLS216 (Dominov et al 1992) and *parA parB parC* (Takahashi et al 1995) from tobacco

Corresponding genomic clones for some of these cDNA clones have been isolated and sequenced These included GmAux28 (pJCW1) and GmAux22 (pJCW2 Ainley et al 1988) and their homologues in *Arabidopsis* (AtAux2-11 and AtAux2 27 Conner et al 1990) SAUR (McClure et al 1989) PS-IAA4/5 PS-IAA6 (Oeller et al 1993) GH3 (Hagen et al 1991) and *ParA and ParB* (Takahashi et al 1995) Soybean GH and SAUR aminoacid sequences were strongly conserved in *Arabidopsis* and pea (Guilfoyle et al 1993)

The cDNA clones of auxin-responsive genes when used as probes demonstrated that auxin can specifically induce the synthesis of these sequences in the species from which they were obtained (Hagen et al 1984 Hagen et al 1988) The auxin-induced genes that have been isolated are divided into two groups (Boot et al 1993) The first group was classified as auxin-responsive genes involved in adaptive responses based on their induction by a variety of stimuli and homology to superfamily of GST These genes include Gmhsp26A GH2-4 *parA parB* pCNT103 107 114 pLS216 A second group of auxin-responsive genes that are induced only by auxin included SAUR GH3 Aux22 Aux28 pIAA4/5 pIAA6 and AGR2

Promoter analysis of various genes regulated by auxin action identified several putative auxin-responsive *cis* elements (Takahashi et al 1995) In pea 194bp sequence of PS-IAA4/5 auxin-responsive gene contained auxin responsive elements (Ballas et al 1993) Liu et al (1994) found two independent 25- and 32-bp auxin-responsive *cis* elements in the GH3 promoter both of which contained the sequence TGTCTC-AATAAG Li et al (1994) showed that a 30bp fragment containing a similar sequence namely TGTCTC in the SAUR promoter region was required for auxin inducibility Takahashi et al (1995) identified two auxin-responsive *cis* elements of 49 and 90bp in the promoter of *parB* gene form tobacco mesophyll protoplasts The presence of these multiple types of *cis* acting auxin responsive elements were suggested to operate in plant cells depending upon both spatial and developmental factors (Takahashi et al 1995)

Studies on mutants altered in auxin metabolism and perception identified auxin-resistant auxin-sensitive and auxin-independent mutants (Barbier-Brygoo 1995) Auxin resistant mutants were isolated based on their ability to grow or proliferate in the presence of toxic levels of auxin These mutants possessed severely affected phenotypes compared to wild type (Barbier Brygoo 1995) Some of the auxin-resistant mutants include *dgt* (Kelly and Bradford 1986) *axr1* (Lincoln et al 1990) *axr2* (Timpte et al 1992) But some of the auxin resistant mutants isolated are also cross resistant to other hormones like *aux1* which was also resistant to ethylene (Pickett et al 1990) and *axr1* which was resistant to cytokinin (Lincoln et al 1990)

Auxin-hypersensitive *aus* 1 mutant (De Souza and King 1991) had suppressed shoot growth and primary root growth and stimulation of secondary root initiation compared to wild type seedling in *Nicotiana Plumbaginifolia*. However this mutant was found to be more ethylene sensitive compared to wild type. Auxin-independent mutants were isolated using T-DNA insertion mutagenesis (Walden et al. 1991). Proliferation of transgenic callus *axi159* isolated after gene tagging with T-DNA from *Agrobacterium* showed auxin independence. Most of the above mentioned mutant genes have not yet been identified except for *AXR1* (Leyser et al. 1993) that encode the ubiquitin-activating enzyme E1. This suggests a role of ubiquitin pathway in auxin action (Barbier Brygoo 1995).

Auxin action on plants has been studied through transformation with genes that modulate auxin levels (Klee et al. 1987). *Agrobacterium tumefaciens* (Ti-plasmid) T-DNA encodes structural genes such as a cytokinin biosynthetic isopentenyl transferase and two IAA biosynthetic enzymes-tryptophan monooxygenase (*iaaM*) and indoleacetamide hydrolase (*iaaH*) that causes tumourous growth in many dicots (Morris 1986). The *iaaM* gene converts tryptophan to indoleacetamide and *iaaH* gene converts IAM into IAA (Klee et al. 1987).

Klee et al. (1987) correlated phenotypic alterations due to changes in the internal auxin levels through engineering *A. tumefaciens* encoded genes for auxin biosynthesis (*iaaM*) in petunia. The internal auxin levels were enhanced tenfold in transgenic petunia plants expressing *iaaM* genes. This resulted in abnormal phenotype such as curling of leaves, and adventitious roots formation. Coexpression of both *iaaM* and *iaaH* genes in tobacco elevated the levels of both free and conjugated auxins (Sitbon et al. 1992). In addition transformation with *A.*

rhizogenes (Ri-plasmid) T-DNA that encodes genes namely *aux1* and *aux2* (Gaudin and Jouanin 1995), *rolA*, *rolB* and *rolC* (Jouanin et al. 1997) induced hairy root syndrome through increased auxin production and increased sensitivity to auxin in dicots (Gaudin and Jouanin 1995; Shen et al., 1988).

Auxin overexpression was performed under the control of different promoters in order to gain a better understanding of the role of auxin in plant morphogenesis and growth. Transgenic plants were produced using *iaaL* (IAA-Lysine synthetase gene of *Pseudomonas syringae* -an enzyme that conjugates IAA and therefore reduces IAA levels) driven by a constitutive promoter in tobacco (Romano et al. 1991). Tobacco plants expressing *iaaL* gene had reduced apical dominance, rooting and also inhibition of vascular differentiation. Plants containing the GH3 promoter fused to the *iaaM* gene from agrobacterium appeared normal most of their life cycle but produced parthenocarpic fruits (Guilfoyle et al. 1993). A similar chimeric gene consisting of SAUR promoter/*iaaM* gene expressing tobacco plants had severe alterations such as 1) adventitious root formation on stem, petioles, 2) curled and narrow leaves, 3) chlorosis and premature senescence 4) dwarfing of plants after a few weeks of growth and 5) hyperlongation of stems and petioles of germinated seedlings grown in light/dark cycles (Guilfoyle et al. 1993).

Transgenic plants with over production or under production of auxins which was under the control of different promoters were crossed with one another in order to gain more information on the role of auxins and the effects of localized expression of auxins. The role of auxin in controlling apical dominance, root growth and vascular differentiation was confirmed by obtaining a phenotypic reversion in plants by crossing plants with the *iaaL* gene and IAA over producers (Romano et al. 1991). Romano et al. (1995) crossed transgenic arabidopsis plants containing *iaaM* with phytochrome deficient *hy6-1* and auxin-resistant *axr1-3* mutants It was found that excess auxin production due to the *iaaM* gene does not counteract inhibition of hypocotyl elongation in hy6-1 seedlings However all of the phenotypic effects due to increased auxin production was suppressed by the auxin-resistant axr1-3 mutations (Romano et al 1995) From these observations, Romano et al (1995) suggested that the *axr 1-3* mutations may have a role in auxin signal transduction since it does not reduce auxin levels when combined with the auxin overproducing transgene

Molecular studies of auxin s role in somatic embryogenesis included isolation and characterization of auxin-responsive genes associated with reactivation of cell division in primary explants by exogenous application of auxin such as 2 4-D northern analysis with auxin-responsive probes transgenic expression studies of promoters regulated by auxins and analysis of changes in gene expression with or without auxin during embryogenesis Auxin induced accumulation of cell cycle specific proteins during reactivation cell division in explants will be dealt with later

The crucial role of auxin in inducing meristematic activity was supported by evidence that the auxin-responsive genes *par* A, *par B* *par* C in tobacco mesophyll protoplast (Nagata and Takahashi 1993) pCNT103 -107 114 and 115 in tobacco cell suspensions (Bnot et al 1993) were found to be expressed during the progression from G0- to S-phase after auxin treatment In addition the *par B* auxin-responsive gene was expressed in the shoot apex and meristematic zone of root tips where distribution of auxin was high (Takahashi et al 1995)

Use of an auxin responsive gene probe as a tool for screening genotypes with greater embryogenic potential has been suggested by Bogre et al 1990 Using such auxin-responsive probes (pJCW1 and pTUC4) Bogre et al (1990) identified that cells of an embryogenic alfalfa genotype were more sensitive to auxin treatment compared to nonembryogenic genotypes

Regulation of auxin-responsive genes during developmental processes involving high cell division activity came from studies with a chimeric construct of T-DNA promoter sequences form Agrobacterium fused to GUS gene The expression of certain T-DNA genes are regulated by phytohormones for example gene 5 of *A tumefaciens* and *aux* gene of *A rhizogenes* are regulated by auxin/cytokinin balances (Gaudin and Jouanin 1995) A short divergent promoter region between the two *aux* gene (encoding two enzymes involved in auxin biosynthesis pathway) coding sequences of *A rhizogenes* strain A4 fused with GUS gene when introduced into tobacco GUS expression was detected in apical meristem root tip meristems lateral root primordia in transgenic calli and cells derived from transgenic protoplasts Based on histological analyses Gaudin and Jouanin (1995) revealed the localization of expression in cells that were reactivated during in vitro culture

Using the GUS gene directed by agrobacterium T DNA gene 5 (whose product converts tryptophan into indole 3 lactate) promoter during somatic embryogenesis Mattsson et al (1995) identified spatial and temporal expression GUS activity at different embryo developmental stages of carrot They showed that GUS expression was confined to the basal part of the embryo at the globular stage In the heart and torpedo stages of embryos GUS activity was detected in the entire embryonic axis but not in the cotyledons During germination high expression was found in the shoot and root apices They correlated GUS activity with high cell division activity in the embryos

The analysis of changes in gene expression through assessment of a protein population detected that there were few changes in protein synthesized between callus cells with auxin and somatic embryos in carrot when auxin was removed (Sung and Okimoto 1981). Kawahara and Komamine (1995) cloned five cDNAs by differential screening of carrot embryogenic cell clusters either in the presence or absence of auxin for 5 days. Expression of one of the clones, CEM 1 (Kawahara et al. 1992) was correlated with cell division activity in the embryo and was expressed preferentially during the globular stage. This CEM1 clone possessed high homology to the EF-1α elongation factor of eukaryotic cells (which is an essential protein in the elongation of peptide chain in protein synthesis) based on the nucleotide sequence and predicted amino acid sequence of the protein (Kawahara and Komamine 1995).

Auxin and Cell Cycle

A reprogramming event of differentiated somatic cells to the embryogenic pathway with auxin (2,4-D) treatment involves a reactivation of the cell division program through coordinated expression of a set of genes and post-translational modifications of regulatory proteins involved in cell cycle control (Dudits et al. 1995). Dudits et al. (1995) proposed an analogy between fertilized egg cell and auxin (2,4-D) treated somatic cells where sperm binding or hormone binding, respectively, trigger the signaling pathway resulting in activation of cell cycle and hence cell division followed by embryogenic differentiation.

With the induction of somatic embryogenesis in carrot cultures, one of the earliest consequences was a decrease in duration of cell cycle or cell doubling time (Warren and Fowler 1979, Fujimura and Komamine 1980). With a leaf mesophyll protoplast that forms a relatively uniform, synchronously responding cell population the relationship between cell cycle resumption and the following embryogenic response were studied (Meyer and Chartier, 1981). Bögre et al. (1990) compared the time course of ^{3}H-thymidine incorporation into leaf mesophyll protoplasts of embryogenic (A2) and non-embryogenic (R15) genotypes of alfalfa in response to 2,4-D treatment. They showed that cell cycle was activated earlier in the embryogenic cultures compared to cultures of the non-embryogenic genotypes. The responses of embryogenic and non embryogenic cells were different when auxin was removed from the medium (Dudits et al., 1995). Embryogenic cells continued division during embryo formation, while the non-embryogenic cells were arrested at the G1 phase by auxin starvation when auxin was removed from the medium (Nishi et al., 1977).

Two major control points in cell cycle are at "start" (where cells become committed to mitotic cell cycle) in late G1 and another at "mitotic initiation" (where timing of entry into mitosis is regulated) in late G2 phase (Nurse, 1990; John et al., 1993). Protein kinase p34^{cdc2}, a cell cycle control protein, is required for G1 to S phase and G2 to M phase transition (Nurse, 1990). It is defined as cell cycle control protein since the mutant form of this protein causes acceleration of cell division in yeast (Lee and Nurse, 1987). Activity of protein kinase p34^{cdc2} catalytic subunit is controlled through association with cyclin proteins (Nurse, 1990) and by phosphorylation and dephosphorylation mechanism by kinase and phosphatase enzyme, respectively, in yeast and animals (John et al., 1993).

Presence of p34^{cdc2} in plants has been identified by its ability to complement yeast cdc2/CDC28 gene and associate with yeast cdc2 control proteins like cyclins (John et al., 1993). Cloning of cyclin genes from carrot (Hata et al., 1991) and alfalfa (Hirt et al., 1992) identified the sequence similarity of plant cyclins with cyclins of other eukaryotes (John et al. 1993). Further, presence of p34^{cdc2} like protein in plants came from the identification of conserved sequence (abbreviated as PSTAIR containing a 34kDA protein) in monocot, dicots, as well as in green, red and brown algae (John et al. 1993). The p34^{cdc2} like protein controls cell cycle by regulating the catalytic activity of protein kinase depending on phytohormonal signals (John et al. 1993); this thus forms one of the essential components of signal transduction during cellular function (Dudits et al., 1995).

In plants, cell division is controlled through regulation of p34^{cdc2} like protein levels (John et al. 1993). Low levels of p34^{cdc2} like protein restrains cell division while higher levels are required for resumption of cell division in meristematic cells (John et al. 1993). Exogenous auxin can cause accumulation of p34^{cdc2} like protein and hence cell division (John et al. 1993). Northern blot analysis with the alfalfa cdc2 cDNA clone revealed the induction and differential expression of this gene in cells of primary root explant, or leaf mesophyll protoplasts in response to 2,4-D treatment (Magyar et al. 1993). However, an additional hormone such as cytokinin is required for stimulation of p34^{cdc2} activity and division in shoot derived tissues (John et al. 1993). In carrot cotyledons and pea roots, auxin treatment alone allowed cells to resume cell division (John et al., 1993). Auxin in tobacco pith and suspension cultures, induced p34^{cdc2} like protein but presence of cytokinin was required for activation and progress of cell division (Gorst et al. 1991; Zhang et al. 1992). Hence phytohormone induced reprogramming of the embryogenic pathway involves regulatory elements in cell cycle control.

Auxin Binding Proteins

Auxin binding protein (ABP) is a kind of hormone receptor protein that binds the auxin (IAA) and initiates a cascade of signal transduction events resulting in a characteristic physiological or biochemical response (Venis and Napier 1995) The earliest report on ABPs came from Hertel et al (1972) where they detected auxin binding activity in microsomal membrane preparations of maize Following this study purification cloning and sequencing led to identification of an auxin binding protein referred as maize ABP1 (Inohara et al 1989)

With photoaffinity labeling studies using tritiated 5-azido-indole-3-acetic acid (Melhado et al 1981) that covalently attaches to ligand binding protein a number of proteins termed as ABP's have been identified in different plant species (Jones and Venis 1989 Hicks et al 1989 Macdonald et al 1991 Zettl et al 1994) However the functions of these proteins as probable specific auxin receptors is questionable except for one or two such as maize ABP1 (Venis and Napier 1995)

A small gene family of at least five members encode various isoforms of maize ABP1 (Venis and Napier 1995) The ABP1 protein sequence consists of 38 hydrobic residues at the N-terminal that constituted a signal peptide for translocation across the endoplasmic reticulum (ER) membrane This signal sequence was followed by 163 residues with a potential N-glycosylation site and a C-terminal KDEL sequence that may signal retention in lumen of the ER (endoplasmic reticulum) (Inohara et al 1989) ABP1 that was glycosylated correctly targeted to the lumen of ER and with auxin binding activity was produced recently via baculovirus expression system (Macdonald et al 1994)

Venis and Napier (1995) suggested that binding of ABP1 to the ligand (auxin) induces conformational changes in ABP1 that results in masking of KDEL sequence. This in turn may result in ABP1 bypassing ER retention signal and continue as a constitutive secretory pathway culminating in a pool of ABP1 on the outside of the plasmamembrane (Venis and Napier, 1995).

Barbier-Brygoo (1995) proposed an auxin perception model at the plasmamembrane in tobacco mesophyll protoplasts involving an interaction between soluble auxin-binding proteins and transmembrane protein (putative candidates are plant receptor like transmembrane kinases) that resulted in a triggering membrane response to auxin.

Auxin-binding proteins have shown to be induced in response to the auxin concentration in the culture medium in microsomal preparations of embryogenic callus lines of carrot (Lo Schiavo et al., 1991). These auxin binding proteins (ABP) are modulated during embryogenesis and organogenesis as shown in carrot and tobacco cultures respectively (Lo Schiavo, 1995). This modulation ABP is reduced or lost when the embryogenic potential is altered or absent (Filippini et al., 1992). Lo Schiavo (1995) suggested that different sets of auxin receptors or ABP's are present in cells that proliferate and in cells that differentiate. Moreover, different auxin receptors may be responsible for different morphogenetic responses (Lo Schiavo, 1995).

In addition to modulation of ABP with different cell types, various genotypes differ in their ABP modulation capacity, i.e., some show all types of auxin receptors within certain nontoxic auxin amounts, while others neither respond to variations in auxin concentration nor modulate ABP's (Lo Schiavo, 1995). Similar results were indicated by a comparative study between embryogenic and non-embryogenic genotypes of alfalfa (Bögre et al 1990 Dudits et al 1995). The protoplasts of a responsive embryogenic genotype of alfalfa were more sensitive to auxin treatment at a concentration that allowed somatic embryo formation without intervening callus growth compared to nonembryogenic type which dedifferentiated but failed to form embryos (Bogre et al 1990).

Concluding Remarks

Over the years somatic embryogenesis has been inducted over 300 species (Bajaj 1995) in a synthetic media supplemented with auxins and cytokinins (dispensable in some systems). The primary explants from various species and tissues respond to hormonal treatments such as 2,4-D by dedifferentiation of the responding tissue. Through this process a new embryogenic developmental program is triggered in differentiated somatic cells by initiation of cell division and reprogramming of gene expression. However a complete understanding of mechanisms of the above process and their regulation during somatic embryogenesis is far from being realized. Is it still unclear as to why different species respond differentially to the presence or absence of auxin and why morphogenic capacity varies with different species, cell types (embryogenic vs non embryogenic) and with aging. A starting point for understanding for molecular basis of auxine involvement in somatic embryogenesis has been provided with identification and involvement of cell cycle protein (p34cdc2-like protein) (John et al 1993) during auxin-stimulated cell division. Furthermore, identification and purification of various auxin receptors and components of the receptor complex (Lo Schiavo et al 1995) will further extend our understanding of morphogenetic capacity and behavior of plant cells Analysis of mutants and transgenic plants could identify key regulatory elements and elements responsible for coordinated gene expression respectively (Dudits et al 1995) Understanding of physiological and molecular basis of somatic embryogenesis can contribute to improve this system for its practical use as a synthetic seed and for somatic hybridization and transgenic research

CHAPTER 3

HISTOLOGICAL COMPARISONS OF SHOOT FORMING AND NON-SHOOT FORMING SOMATIC EMBRYOS OF SWEETPOTATO

(*Ipomoea batatas* (L) Lam)

Introduction

The process of somatic embryogenesis is a unique feature in plants (Zimmerman 1993) Over the years somatic embryogenesis has been induced in many agronomically and horticulturally important species through manipulation of cultural conditions Most research on somatic embryogenesis has concentrated on developmental embryogenesis except for a few studies related to areas such as plasticity in morphogenetic patterns of a somatic embryo (Ammirato 1977 1985 Schiavone and Cooke 1987) and developmental patterns of somatic embryo variants leading to conversion into plantlets (Chee and Cantliffe 1988 Nickle and Yeung 1993)

Morphogenetic processes in plant embryo development are thought to be highly conserved The process proceeds through globular heart torpedo and cotyledonary stages suggesting a developmental regulation of gene expression (Borkird et al 1988) "Conversion" is a process that refers to shoot meristem formation and subsequent vegetative leaf initiation (Nickle and Yeung 1994 Merkle et al., 1987)

The development of somatic embryos in culture is considered to be "plastic" (Ammirato 1985) A variety of somatic embryonic forms may develop during embryogenesis They may have a great variability in their developmental patterns and conversion to plantlets (Ammirato 1985 1987 Chee and Cantliffe 1988) Chee et al (1990) suggested that the selection of the proper embryo developmental stage as a prerequisite for improved germination and subsequent plant formation in sweetpotato Chee and Cantliffe (1988) and Schultheis et al (1990) identified the torpedo cotyledonary and elongated torpedo stages as three potential developmental stages of somatic embryos which had the ability to convert into plantlets in sweetpotato In orchard grass Gray et al (1987) suggested that embryos with well developed scutellar and coleoptilar regions converted better However Kerns et al (1986) and Merkle et al (1990) reported that many well formed somatic embryos fail to form plantlets Therefore morphologic variants exists even among the torpedo and cotyledonary stage embryos that are mature enough to give rise to plants (Chee and Cantliffe 1988) Even though such morphologic variants of somatic embryos appear normal externally they may be abnormal internally in terms of cellular and tissue differentiation (Ammirato 1985) Ammirato (1987) attributed failure of embryos to form shoot to the malformation of shoot apical meristem that starts developing during the torpedo stage A comparison of external morphology with internal anatomy of somatic embryos with morphogenetic variants is necessary and long overdue in order to understand and elucidate different developmental pathways in embryogenesis Such comparisons may identify which variants best form plantlets This information can then be used for synthetic seeding systems or transgenic research

A machine vision system serves as a tool to identify and classify biological material without interfering with its aseptic environment (Molto and Harrell, 1992; Harrell et al., 1992). A machine vision system for embryo and callus identification consists of a 32 bit computer equipped with three image acquisition cards, a color video camera and a stroboscopic illuminator (Cantliffe et al., 1993) that captures the images of the embryos without affecting the aseptic environment. The purpose of the present study was to elucidate the developmental pathways of somatic embryos by comparing external morphology (captured via machine vision) with internal anatomy in order to identify the shoot forming and non-shoot forming morphogenic variants. Torpedo and cotyledonary stages of sweetpotato somatic embryos in were used as the test materials.

Materials and Methods

Basal medium consisted of a semisolid medium with the inorganic salts of Murashige and Skoog (1962), 500µM myo-inositol, 5µM thiamine HCl, 10µM nicotinic acid, 5µM Pyridoxine HCl, 3% (w/v) sucrose and 0.7% Phytagar® (Gibco) (Chee and Cantliffe, 1989). The medium pH was adjusted to 5.8 using 1N sodium hydroxide prior to autoclaving at 121 C for 15 min. Twenty ml of medium was dispensed into presterilized 100 x 15mm plastic Petri dishes.

Embryogenic Culture Initiation and Maintenance

Apical and axial shoot tips of *Ipomoea batatas* were used to initiate embryogenic cultures as described by Liu and Cantliffe (1984). Embryogenic callus was gently broken with a sterile glass slide tip and then sieved through mesh sizes

710µm and 355µm The callus collected on each sieve was rinsed with 3% (w/v) sucrose solution The resultant 355-710µm fraction was plated on to callus proliferation medium containing modified MS basal medium as described above with 30mM KCl 10µM 2 4D and 1µM BA (Chee and Cantliffe 1992) The cultures were maintained in the dark at 30°C in 8 weeks cycle

Embryo Production and Harvest

Mature somatic embryos can arise from callus maintained in the auxin containing callus proliferation medium without the transfer to auxin-free medium Mature somatic embryos consisting of torpedo and cotyledonary stages could be harvested at 7 weeks of callus age in callus proliferation medium Embryos were harvested using sterile forceps under Nikon stereomicroscope and were transferred individually onto small Petri dishes (60x15mm) containing embryo conversion medium for root/shoot conversions Embryo conversion medium consisted of basal medium as described above with 1 6% (w/v) sucrose and no growth regulators (Chee et al 1992)

Machine Vision and Histological Analyses

Harvested mature somatic embryos were subjected to a machine vision analysis system (Harrell et al 1992) on the day of harvest (day 0) as described in chapter 4 Seventy five embryos were subjected to histological analyses (Jensen 1962) Five embryos were removed from culture after 1 5 10 15 20 days and fixed in FAA and dehydrated through ethanol and tertiary-butanol series (Jensen 1962) These samples were paraffin embedded and sectioned using a rotary microtome The sections were affixed to glass slides smeared with Haupt's adhesive along with 2% formalin on a warming plate at 50-55°C These slides were placed in an oven at 45 C for 24 hours Paraffin sections were removed and washed using a xylene and ethanol series and sections were stained with toluidine blue (Jensen 1962) After washing and clearing with the ethanol series and xylene the slides were covered with cover slip permount^{TM} (Jensen 1962) The experiment was repeated three times Serial sections of the embryos were studied and photographed using a Nikon compound microscope

Zygotic embryos were excised from *I batatas* seeds (unknown genotype) Seeds were first immersed in concentrated sulphuric acid for 15 min and then rinsed with water to soften the hard seed coat Softened seeds were placed in a glass Petri dish on filter paper moistened with water and embryos were excised after 12 24 hr incubation (Chee & Cantliffe 1988) Zygotic embryos were sectioned using the procedure mentioned above for somatic embryos to have background information on zygotic embryo anatomy for comparison with somatic embryo sections

Scanning Electron Microscopy

Scanning electron microscopical examinations of embryos were done to look at external morphology of the embryo especially at shoot axis Shoot forming and non-shoot forming somatic embryos were fixed in 2 5% glutaraldehyde and 0 1M cacodylate buffer and dehydrated through an ethanol series and critical point dried The embryos were mounted on aluminum stubs sputter coated with gold/palladium and examined under Hitachi S-450 scanning electron microscope

Results

Scanning Electron Microscopy

Scanning electron microscopic examinations of the shoot apical region on embryos after a 20 day period in conversion medium (Figs 3-1 A B C D E) indicated that shoot forming and non-shoot forming embryo morphologies were distinctly different in the shoot apex region Shoot forming embryos had a well developed and wider shoot apex that allowed developing leaf primodia to emerge The non-shoot forming embryos looked normal externally with or without cotyledon development. The orifice at the shoot apex region was however too narrow to allow any shoot primodia development and emergence Root development appeared normal in shoot and non-shoot forming embryos (Fig 3-1 F)

Histological Studies

Longitudinal sections of each embryo that was sectioned at different stages after transfer to conversion medium were compared with their respective external morphological images stored in the computer (vision system) This comparison of external morphology and internal anatomy of the embryos identified five morphological variant types among the torpedo and cotyledonary stage embryos These morphological variant types included

(a) **Perfect Type** (Fig 3-4)

(b) **Near Perfect Type** (Fig 3-5)

(c) **Limited/No Apical Meristem Activity Type** (Fig 3-6)

Fig 3-1 Shoot forming and non-shoot forming embryo morphologies of sweetpotato somatic embryos

A B C Scanning electron micrograph of shoot apex region of shoot forming embryo variants of sweetpotato D E Scanning electron micrograph of shoot apex region in non-shoot forming embryo variants of sweetpotato with very narrow shoot apical pore (arrows) F Scanning electron micrograph of a well-developed root of a somatic embryo of sweetpotato

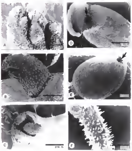
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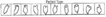
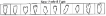
Fig 3-2 Morphological fate map of different morphological variants of somatic embryos of sweetpotato

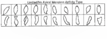
Perfect Type



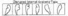
Near Perfect Type



Limited/No Apical Meristem Activity Type



Disrupted Internal Anatomy Type



Proliferating Type

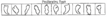


Fig 3-3 Zygotic embryo of sweetpotato

A, External morphology of a zygotic embryo of sweetpotato. B. Light micrograph of internal anatomy of the zygotic embryo of sweetpotato sectioned after 12-24 hr incubation on a filter paper moistened with water. (Bar in white=1mm. Bar in black=0.1mm)

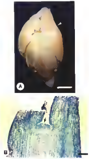

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Fig 3-4 Perfect Type somatic embryos of sweetpotato

External morphology of 75 somatic embryos were captured via computer vision analysis on the day of harvest. Five embryos were selected after 1 5 10 15 20 in culture and then subjected to histological analyses A C E External morphology of Perfect Type embryo variants in sweetpotato with well developed cotyledons that are either single or bi-lobed (arrows) B Light micrograph of internal anatomy of a Perfect Type somatic embryo (A) after 5 days in conversion medium with a well developed shoot apical meristem (arrow) D Light micrograph of internal anatomy of a Perfect Type somatic embryo (C) after 10 days in conversion medium with a developing leaf primordia (arrow) F Light micrograph of internal anatomy of a Perfect Type somatic embryo (E) at day 1 in conversion medium with a well formed shoot apical meristematic dome (arrow) (Bar in white = 1mm Bar in black =0 1mm)

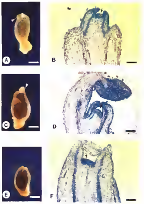
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Fig 3-5 Near Perfect Type somatic embryos of sweetpotato

External morphology of 75 somatic embryos were captured via computer vision analysis on the day of harvest Five embryos were selected after day 1 5 10 15 20 in conversion medium and then subjected to histological analyses A C External morphology of Near Perfect Type embryo variants in sweetpotato (a (arrow) distinctive constriction at the shoot apical notch c (arrow) cotyledon development that are either single or fused) B Light micrograph of internal anatomy of Near Perfect Type somatic embryo (A) at day 1 in the conversion medium with development of at least a bilayer of meristematic cells (arrow) D Light micrograph of internal anatomy of Near Perfect Type somatic embryo (C) after 5 days in conversion medium with well developed of shoot apical meristematic dome (arrow) (Bar in white= 1mm Bar in black=0 1mm)

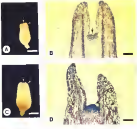
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Fig 3-6 Limited/No Apical Meristem Activity Type somatic embryos of sweetpotato

External morphology of 75 somatic embryos were captured via computer vision analysis on the day of harvest Five embryos were selected after day 1 5 10 15 20 in culture and then subjected to histological analyses A C External morphology of Limited/No Apical Meristem Activity Type embryo variants in sweetpotato (a (arrow) no distinctive constriction at the shoot apex c (arrow) no or rudimentary cotyledon development that are either single lobed or fused) B Light micrograph of internal anatomy of Limited/No Apical Meristem activity Type embryo (A) at day 1 in the conversion medium with no shoot apical meristem activity (arrow) D Light micrograph of internal anatomy of Limited/No Apical Meristem activity Type embryo (C) after 20 days in conversion medium with no shoot apical meristem activity (arrow) (Bar in white= 1mm Bar in black=0 1mm)

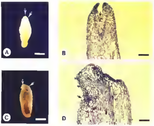
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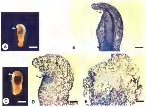
Fig 3-7 Disrupted Internal Anatomy Type somatic embryos of sweetpotato

External morphology of 75 somatic embryos were captured via computer vision analysis on the day of harvest Five embryos were selected after day 1 5 10 15 20 in culture and then subjected to histological analyses A C External morphology of Disrupted Internal Anatomy Type embryo variants in sweetpotato with a side shifted shoot apical notch (arrow) B Light micrograph of internal anatomy of Disrupted Internal Anatomy Type somatic embryo (A) at day 1 in conversion medium with no shoot apical meristem activity (a arrow) D E Light micrograph of internal anatomy of Disrupted Internal Anatomy Type somatic embryo (C) after 15 days in conversion medium with resumption of some shoot apical meristematic activity (b arrow) and disruption of vascular tissue (c arrow) (Bar in white= 1mm Bar in black=0 1mm)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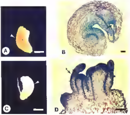
E

Fig 3-6 Proliferating Type somatic embryos of sweetpotato

External morphology of 75 somatic embryos were captured via computer vision analysis on the day of harvest. Five embryos were selected after day 1 5 10 15 20 in conversion medium and then subjected to histological analyses in 3 replications. A C. External morphology of Proliferating Type embryo variants in sweetpotato with a typical curvature in the expanded hypocotyl (arrow). B. Light micrograph of internal anatomy of Proliferating Type somatic embryo (A) after 5 days in conversion medium with flattened shoot apical meristem (a arrow). D. Light micrograph of internal anatomy of Proliferating Type somatic embryo (C) after 10 days in conversion medium with multiple meristematic activity at the shoot apex (b arrow). (Bar in white= 1mm Bar in black=0 1mm)


A
B
C
D

(d) **Disrupted Internal Anatomy Type** (Fig 3-7)

(e) **Proliferating Type** (Fig 3-8)

Of these different type embryos Perfect Type and Near Perfect Type embryos constituted about 40% of the embryos of the total population The Limited/No Apical Meristem Activity Type embryo Disrupted Internal Anatomy Type Embryo and Proliferating type embryo constituted the remaining 60% of the total population of the embryos (Fig 3-2) with majority of the embryos (about 34%) categorized under Limited/No Apical Meristem Activity Type

Perfect Type

The Perfect Type embryo consisted of mostly cotyledonary stage embryos The cotyledonary embryo morphological variants included narrow and thin as well as wide and thick types and were about 2 3mm long The cotyledons in these embryos where either single lobed or two lobed and were sometimes fused (Figs 3-4 A C E) However cotyledons comparable to zygotic embryo cotyledons were not found in zygotic embryos cotyledons were long bi-lobed and folded like wings (Fig 3-3 A) Cotyledonary stage somatic embryo were characterized by a vase shape (Chee and Cantliffe 1988) and possessed a constriction at the shoot pole

Internal anatomy of these Perfect Type embryos contained dense cytoplasmic cells in the shoot apical notch and well developed vascular tissue (Figs 3-4 B D F) comparable to the zygotic embryo sections (Fig 3-3 B) Meristematic cells with of shoot apical region with dense cytoplasm first elevates into a dome shape before the formation of shoot primodia (Nickle and Yeung 1993) Cotyledonary stage somatic embryo variants possessed a dome shaped shoot apical meristem at the distal portion of the shoot apex even at day 1 in conversion medium (Fig 3-4 F) With passage in the conversion medium they gave rise to leaf primodia within 5 days in culture (Fig 3-4 B) These embryos also contained a well developed root meristem In sweetpotato most of the embryos of all types gave rise to roots and had a well developed root meristem

Near Perfect Type

The Near Perfect Type embryo consisted of mostly torpedo to late torpedo stage embryos The torpedo embryo morphological variants were usually long and thin, creamish white color with rudimentary cotyledon development or fused cotyledons (Fig 3-5 A C) There was a distinct constriction at the shoot apex in these embryos

Longitudinal sections of these Near Perfect Type embryos maintained at least a bilayer of dense cytoplasmic cells with few vacuoles (Fig 3-5 B) at day 1 in culture medium These cells divided and resumed a meristematic dome after 5 10 days in culture (Fig 3-5 D) and gave rise to a shoot after 10 -15 days total in culture These embryos also sustained a well developed vascular tissue and root meristem

Limited/ No Apical Meristem Activity Type

The Limited/ No Apical Meristem Activity Type embryo consisted of mainly torpedo to late torpedo stage variants These variants were usually long and thin inverted cone shaped with a closed apical end that was either smooth or rounded without any constriction or depression and possessed rudimentary or no cotyledon development (Fig 3-6 A, C) Some variants also included vase shaped embryos with a very thin and long neck like hypocotyl

The internal anatomy of these embryos revealed that cells in the shoot apex which were usually densely cytoplasmic but became vacuolated indicating no apical meristem activity in the embryo at day 1 in culture (Fig 3-6 B) Even after 20 days in culture when in most of the embryos converted to plantlets there was no resumption of meristematic activity at the apex (Fig 3-6 D)

Disrupted Internal Anatomy Type

The Disrupted Internal Anatomy Type embryo characterized mostly torpedo stage embryos These torpedo embryo morphological variants were usually small and kidney shaped yellowish with some grey color discoloration in the interior of the embryo (Fig 3-7 A C) These embryos were typified by the presence of an apical notch shifted towards one side of the embryo with single or no rudimentary cotyledon development

Examinations of serial sections of these types of embryos revealed good vascular tissue development at day 1 in culture but evidence for the presence of at least a bilayer of meristematic cells was lacking (Fig 3-7 B) As they continued to grow in the conversion medium vascular tissue was completely disrupted with premature differentiation and distortion of cell lineage especially in the cortex at day 15 in culture (Fig 3-7 D) There was recapitulation of some meristematic activity after 15 days in culture in the side shifted apical notch in some embryos (Fig 3-7 E) However no visible shoot development occured in these embryos even when they were allowed to develop for a longer time in conversion medium These embryos started to turn brown externally in about 15 days in culture and eventually ceased to grow

Proliferating Type

Late torpedo and cotyledonary stage embryo variants constituted the Proliferating Type. These variants contained a definite smooth curvature in the expanded hypocotyl region (Fig. 3-8 A, C). Some of the embryos were curved on both sides of the hypocotyl resembling a dumb bell shape. The cotyledons in these embryos were either single lobed or two lobed or sometimes fused (Fig. 3-8 A, C).

Longitudinal sections exhibited multiple meristematic region through out these embryos (Fig. 3-8 B) and a flattened apical meristem at day 5 in culture and at the shoot apex (Fig. 3-8 B) at day 15 in culture, resulting in external callus formation. Along with callus proliferation either at the root pole or both the root and shoot pole, some of the embryos gave rise to multiple organized shoots. However, these embryos retained well developed vascular tissue and root meristem.

Discussion

Somatic embryogenesis has shown to produce imperfect embryos with abnormalities ranging from connated or fused cotyledons, single cotyledon, multiple cotyledons, secondary embryogenesis at the radicle pole, precocious vacuolation and cell differentiation (Ammirato, 1985; Gatau et al., 1991; Merkle et al., 1990; Nickle & Yeung, 1993). Lack of conversions was traced to anomalies in the shoot apical meristem (Ammirato, 1985; Merkle et al., 1990). These anomalies included failure to establish a functional shoot meristem (Nickle & Yeung, 1993), premature conversion, extended cell divisions in the shoot apex (Ammirato, 1985) and precocious vacuolation in the shoot cortex, or cotyledon abnormalities (Fujii et al.

1990 Quinn et al 1989 Merkle et al 1990) Nickle and Yeung (1993 1994) found that loss of conversion capacity in carrot embryo cultures was due to abnormal development of the shoot apical meristem followed by abortion of the meristem

These findings present at least five different developmental pathways leading to competence or noncompetence of somatic embryos Among the morphological variants of torpedo and cotyledonary stage embryos the "Perfect" and "Near Perfect" (Fig 3-4 and 3-5) types were competent to produce whole plants (by comparison of external morphology and internal anatomy) The other three types namely "Limited/No Apical Meristem Activity Type"(Fig 3-6) "Disrupted Internal Anatomy Type"(Fig 3-7) and "Proliferating Type"(Fig 3-8) were found noncompetent with respect to their ability to give rise to whole plants

For all morphological somatic embryo variants in sweetpotato shoot formation was much less than root formation which led to low plant recovery (see Chapter 4) This indicated that the shoot meristem was not as well organized or developed as the root meristem In sweetpotato the lack of organized shoot development was attributed to the following abnormalities 1) lack of an organized apical meristem (Fig 3-6 B) 2) sparsity of dividing cells in the apical region (Figs 3-6 D & 3-7 A D) 3) flattened apical meristem (Fig 3-6 B) 4) multiple meristemoids and/or diffuse meristematic activity throughout the embryo (Fig 3-8 B D) Out of these four abnormalities for lack of organized shoot formation the first two categories constituted about 80% of the embryos while the third and fourth categories constituted about 20% of the embryos

A variety of somatic embryonic forms may develop during embryogenesis They may have a great variability in their developmental patterns and conversion to plantlets (Ammirato 1985, 1987; Chee and Cantliffe 1988). Auxin appears to have a role not only in these sequences that precede embryo formation, but also subsequent morphogenic events in embryo development (Michalczuk et al. 1992 a, b; Zimmermann 1993). Exogenously applied synthetic auxin such as 2,4-D stimulates the accumulation of endogenous IAA (indole acetic acid) (Michalczuk et al. 1992b). This maintains the proliferative state of the callus and prevents subsequent embryo formation (Michalczuk et al. 1992b). Transferring the tissue to a 2,4-D free medium results in the decline of total IAA to levels low enough to set up an internal gradient for initiation and maintenance of polarized growth and subsequent embryo development (Michalczuk et al. 1992a).

Studies by Schiavone and Cooke (1987) and Liu et al. (1993) have revealed the importance of proper polar auxin transport for normal morphogenesis once embryogenesis has been induced. Schiavone and Cooke (1987) showed that treatment of embryos with inhibitors of polar auxin transport prevented their development to the next morphogenic stage, i.e. the globular and oblong embryos (intermediate stage between globular and heart) to oblong and heart stages, respectively. Instead these embryos were shown to increase in size without cotyledon initiation. When polar auxin transport was blocked in cultured zygotic embryo of Indian mustard at globular stage, fused collar like cotyledons formed (Liu et al. 1993). The embryos resembled pin-1 mutant of *Arabidopsis*, which has reduced auxin transport in its inflorescence (Okada et al. 1991). Some of the Near Perfect and Limited/No Apical Meristem Activity Type in sweetpotato somatic embryos (Fig. 5-2) possessed fused collar like cotyledons resembling the Indian mustard experiment by Liu et al. (1993). In addition, the Limited/No Apical Meristem

Activity Type resembled the apical domain mutant *gurke* that lacked cotyledons and shoot apical meristem (Mayer et al 1991)

Low conversion rate of somatic embryos may limit its application in synthetic seed technology (Janick et al 1989 Lakshmana and Singh 1991) Evidence presented here and in other studies points towards the abnormality in the shoot apical meristem as the main cause for lower conversion rate Recently Nickle and Yeung (1993 1994) reported that failure to establish functional meristem in carrots could be due to precocious initiation of germination events such as excessive vacuolation in the shoot apical region They showed that through emulation of in ovulo environment i.e by application of ABA considerable cytoplasmic retention in the shoot apical meristem was achieved conserving its capacity to form a shoot. Further early embryo stages were more responsive compared to mature embryos However in yellow-poplar somatic embryos treated with ABA embryos appeared well formed and mature but failed to convert (Merkle et al (1990) The authors suggested that this was due to residual effects of ABA Identification of conversion competent embryos is very essential for improved regeneration efficiency regardless of what hormone(s) is used to initiate shoot meristems

Somatic embryos are manually sorted by a human expert into competent and noncompetent classes based on visual cues before the transfer to conversion medium Straut et al (1985) suggested that morphological features serve as an identifiable marker in determining conversion competent embryos Embryo length has been positively correlated with germination and/or plant formation in zygotic and somatic embryos (Gray et al 1987 Stuart et al 1985) However heterogeneity even among torpedo and cotyledon stage somatic embryos effects the performance of a human expert in identifying conversion competent from noncompetent embryos

Torpedo and cotyledonary morphologic variants of somatic embryos may appear normal externally, but may be abnormal internally in terms of cellular and tissue differentiation (Ammirato 1985). This further complicates the sorting of embryos.

In order to improve recognition and sorting of embryos, human recognition of competent somatic embryos based on visual observations was mimicked using a computer vision analysis in this study. Information relating the internal anatomy of torpedo and cotyledonary embryo variants to their external morphology can help further improve sorting efficiency. Identification of the various morphological variants of somatic embryos that convert to plantlets is a major step for application of this process for "synthetic seed" technology (Chee and Cantliffe 1988) and improved plant regeneration for transgenic research.

Summary

A comparison of external morphology captured via a machine vision system and a study of internal anatomy of the somatic embryos identified five different major morphological variants among torpedo and cotyledonary stage embryos. These included 1) Perfect type, 2) Near Perfect type, 3) Limited/No Meristematic Activity type, 4) Disrupted Internal Anatomy type, 5) Proliferating Type. Perfect and Near Perfect types of somatic embryos were categorized as competent, while Limited/ No Meristematic activity, Disrupted Internal Anatomy, Proliferating types were categorized as noncompetent with respect to their ability to convert into plantlets. Lack of organized shoot development in somatic embryos of sweetpotato was associated with the following abnormalities: 1) lack of an organized apical meristem

2) sparsity of dividing cells in the apical region 3) flattened apical meristem 4) multiple meristemoids and/or diffuse meristematic activity throughout the embryo The morphological fate of most torpedo and cotyledonary embryo variants was mapped which will be beneficial in synthetic seeding and transgenic research and development of sweetpotato

CHAPTER 4
COMPUTER VISION ANALYSIS OF SOMATIC EMBRYOS FOR ASSESSING THEIR CONVERSION TO PLANTLETS

Introduction

Sweetpotato, a valuable source of food and biomass, is conventionally propagated through stem cuttings (Cantliffe, 1992). However, this propagation method is not cost effective because of high labor costs, the need for maintenance and multiplication of virus-free stocks, requirement of large nurseries, and lack of storage facilities for mass production (Cantliffe, 1992). Alternately, in vitro culture offers an excellent propagation technique and is effective in obtaining disease free stocks (Cantliffe, 1992). In sweetpotato, mass production of somatic embryos to use as synthetic seeds and subsequent direct seeding would facilitate a significant reduction of production costs for this vegetatively propagated crop (Cantliffe, 1992).

Somatic embryogenesis of sweetpotato produces mature embryos, which convert into plantlets on basal nutrient medium. Sweetpotato somatic embryos mimic their zygotic counterparts in the formation of globular, heart, torpedo and cotyledonary developmental stages. The torpedo and cotyledonary stage embryos have potential to be used as functional seeds referred as "synthetic seeds" (Gray and Purohit, 1991). Synthetic seeds have wide application including micropropagation, germplasm storage and plant breeding (McKersie et al., 1989). Somatic embryogenesis is not only important for regeneration of plants but also serves as a tool for understanding the regulation of gene expression and morphogenesis in early embryogenesis (Zimmerman 1993)

Although somatic embryogenesis can be induced in many species its potential as an automated clonal propagation method has not yet been fully achieved This is mainly due to poor conversion rate to plantlets owing to poor embryo quality and maturity

The main problems that are encountered in automation of somatic embryogenesis are asynchronous development, formation of aberrant or malformed embryos with malformed or suppressed cotyledon development and incomplete or lack of maturation (Stark and de Vries 1993) As embryos mature they often germinate precociously without undergoing desiccation and quiescence (Kermode 1990) The most critical step in the production of synthetic seed is acquisition of desiccation tolerance which will enable storage for a long period of time (McKersie and Bowley 1993) Florin et al (1993) suggested that only dried somatic embryos could have a future for direct sowing under field conditions by farmers Further incorporation of various additives around the dry embryo to provide nutrients mechanical protection and other supplements such as fungicides *Rhizobium* or pesticides would be required for successful emergence of plantlets and implementation of synthetic seeding (McKersie and Bowley 1993)

Application of somatic embryos as synthetic seeds requires a fairly homogenous population of competent embryos with a uniform and high rate of conversion However this is very difficult to achieve because the population of somatic embryos in culture is highly heterogeneous Among torpedo and cotyledonary embryo stages there are commonly variations related to size shape and color. This challenges the biological researcher to identify competent embryos which would give rise to plants. A machine vision system that serves as a tool to identify and classify biological material without interfering with its aseptic environment (Molto and Harrell 1992, Harrell et al 1991) could be used to select competent somatic embryos (Harrell et al 1994). A machine vision system has been developed which consists of a 32 bit computer equipped with three image acquisition cards, a color video camera and a stroboscopic illuminator (Cantliffe et al 1993). This machine vision system captures the images of the embryos without affecting the aseptic environment. Use of computer vision analysis to qualify and quantify research in somatic embryogenesis process has been demonstrated previously (Grand d'Esnon et al 1989, Cazzulino et al. 1990). Cazzulino et al (1988) developed a model for carrot somatic embryo growth by estimating the number and developmental stage of embryos in suspension.

Grand d'Esnon et al (1988) distinguished nonembryogenic from embryogenic callus using differential coloring of the vacuolated non embryogenic callus (grey) compared to cytoplasmic embryogenic callus (black) when black lighted. They also were able to select torpedo stage embryos using template matching algorithms. Automated recognition solely based on morphological features of different developmental stages of somatic embryos was demonstrated in birch (Hamalainen et al 1993). However a comparison of visual morphological observation with the subsequent conversion of selected embryos into plantlets was not demonstrated in these studies.

The overall goal of the present work was to improve embryo regeneration efficiency by identifying viable conversion competent embryos. Identification of competent and noncompetent classes of somatic embryos in *Ipomoea batatas* was accomplished using a machine vision analysis system Size shape and color related measurements extracted using the machine vision system were used to classify the embryos following the Discriminant Analysis and Decision Tree Analysis These results were compared with the conversion results in order to test the performance of the system

Materials and Methods

Basal medium consisted of a semisolid medium with the inorganic salts of Murashige and Skoog (1962) 500µM myo-inositol 5µM thiamine Hcl 10µM nicotinic acid 5µM pyridoxine HCl 3% (w/v) sucrose and 0 7% Phytagar® (Gibco) (Chee and Cantliffe 1989) Prior to autoclaving at 121°C for 15 min pH was adjusted to 5 8 using 1N sodium hydroxide Twenty ml of medium was dispensed into presterilized 100x15mm plastic petri dishes

Embryogenic Culture Initiation and Maintenance

Apical and axial shoot tips of *Ipomoea batatas* were used to initiate embryogenic cultures as described by Liu and Cantliffe (1984) Embryogenic callus was gently broken with a sterile glass slide tip and sieved through mesh sized 710µm and 355µm The callus was collected on each sieve and was then rinsed with 3% (w/v) sucrose solution The resultant 355-710µm fraction was plated onto callus proliferation medium containing modified MS basal medium as described above with 30mM KCl 10µM 2 4D and 1µM BA (Chee and Cantliffe 1992) The cultures were maintained in the dark at 30°C in an 8 week-transfer-cycle

Embryo Production and Harvest

Somatic embryos arise from callus maintained in auxin containing callus proliferation medium without the transfer to auxin free medium. Somatic embryos consisting of torpedo and cotyledonary stages could be harvested at 7 weeks of callus age in callus proliferation medium. Harvested embryos were transferred individually onto embryo conversion medium in small petri dishes (60x15mm). The embryo conversion medium consisted of basal medium as described above with 1.6% (w/v) sucrose (Chee et al., 1992).

Machine Vision and Data Analysis

Mature somatic embryos (378) were harvested and subjected to machine vision analysis (Harrell et al., 1992) on the day of harvest (day 0). The machine vision system consisted of a zoom stereomicroscope (Nikon SMZ 2T), a color video camera (Pulnix TMC-54G), a 32-bit computer (Mizar 7120) equipped with three image acquisition cards (Datacube VVG128), and a custom Stroboscopic Illuminator (Harrell et al. 1992, 1993). Red-green-blue video signals generated by the camera at a 5-bit resolution were digitized using the three image acquisition cards (Harrell et al. 1992). A nonblurred image of each embryo was created by synchronizing the image acquisition with the flash of the stroboscopic illuminator (Harrell et al. 1992, 1993, 1994). Images of 378 embryos in total from three individual experiments were archived and saved in the computer before the embryos were transferred on to a conversion medium. The embryo size and shape related features from the saved embryo images were extracted using 32 radii length or distances from the embryo centroid to 32 perimeter points at constant angular increments (Harrell et al. 1993 and Harrell et al 1994) The color related features such as hue intensity and saturation were extracted using a color segmentation algorithm developed by Harrell et al (1987)

Seventy five embryos were selected at random and subjected to histological analyses (see chapter 3) at days 1 5 10 15 20 Based on their internal anatomical characteristics and external root/shoot conversions the embryos were classified as competent or noncompetent (see chapter 3) The remaining 303 embryos were subjected to a 20 day conversion study and were scored for root and shoot conversions at day 1 5 10 15 20 in the conversion medium An embryo was scored competent only if it possessed both a shoot and a root, otherwise it was scored noncompetent.

Size shape and color features extracted out of the 378 embryo images using the machine vision system were subjected to Fisher discriminant analysis (Fisher 1938) and Decision Tree classifier system (Gordon 1987) For statistical purposes the size shape and color feature measurements of 378 embryos images were subdivided at random into 3 sets namely

1 **Original set** consisted of 75 embryos (which were also subjected to anatomical studies (see Chapter 3) The purpose of the "original set" was to develop the discriminant function.

2 **Training set** consisted of 152 embryos which were subjected to conversion studies The purpose of the "training set" involved training of developed discriminant function (Her et al 1987) from the "original Set "

3 **Classification set** consisted of 151 embryos which were subjected to conversion studies The purpose of the "Classification set" was to test the validity of the discriminant function (Hair et al 1987)

Results

Conversion Results

Most embryos produced roots compared to shoots after 20 days while only half of the embryos produced both roots and shoots (Table 4-1) In about 5% of the embryos which formed both roots and shoots callus proliferation also occured mostly near their root poles (Table 4-1) Within 10 days in conversion medium most of the embryos had formed roots Shoot conversion occurred after 10-15 days in culture medium (Fig 4-1)

Somatic Embryo Classification

A Discriminant Analysis

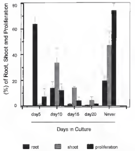
In this study 7 size descriptors 10 shape descriptors and 3 color descriptors were measured Size variables included area average radius length width elongation radius variation and elongation Shape variables included asymmetric major asymmetric minor asymmetric ratio roughness box-fill circle-2 side variation slope variation and parallel Color variables are hue saturation and intensity of the centroid cotyledon and radicular region of the embryo. Detailed description of each of the above variables are reported in Harrell et al (1993 1994) (see appendix)

Table 4-1 Conversion results, root, shoot and proliferation in embryos occuring after 20 day conversion study

Criterion	Conversion† (% ± SE)
Root	79.7 ± 4.5
Shoot	50.6 ± 5.6
Root and Shoot	43.2 ± 5.6
Root, Shoot and Proliferation	4.6 ± 2.4

† Percentage of conversion ± standard error

Data obtained from 304 somatic embryos pooled from three experiments after a 20 day conversion study



Never represents occurence of no root shoot and or proliferation after 20 day culture period

Fig 4-1 Root shoot and proliferation in embryos occuring over a 20 day culture period

Descriptive statistics (Table 4-2, 4-3) identified the differences in the averages of size variables, shape variables and color variables among competent and non-competent classes. In order to help distinguish the existence of any significant differences in the size, shape and color variables among competent and noncompetent classes of somatic embryos, univariate F tests (Ott, 1993) were performed with these measurements in the original or analysis set. With 2 groups, namely competent and noncompetent, an ANOVA with an F-test is the same as a two sided t test (Ott, 1993). Area, average radius, length, and width, in case of size variables (Table 4-4) were different between competent and noncompetent classes. In the case of shape variables (Table 4-5) only asymmetric minor (refers to asymmetry with respect to the minor axis) exhibited differences.

There were differences in color observations such as the saturation and intensity of radicular, cotyledonary and centriod regions of competent and noncompetent classes of somatic embryos (Table 4-6).

The results of univariate analysis (Ott, 1993) revealed that there were differences with respect to morphological parameters between competent and noncompetent classes of somatic embryos when one variable at a time was considered. However, results of the preceeding analyses neither revealed the differences between the competent and noncompetent embryos (when all the variables considered together) nor did they explain the interrelationship between these variables, for example among size variables, etc. In such cases, multivariate analysis (Hair et al., 1987) such as Discriminant Analysis, also referred as "Fisher Discriminant Analysis" (Fisher, 1936; Cruz-Castillo et al., 1994) or "Canonical Discriminant Analysis" (Rencher, 1992; Cruz-Castillo et al., 1994) could be applied.

Table 4-2 Descriptive statistics of competent class of somatic embryos from the Original or Analysis set.

Feature†	Mean	Std Dev
Area(μm^2)	3058412.04	1189676.81
Average radius(µm)	1020.69	211.30
Length(L)(µm)	2990.04	739.72
Width(W)(µm)	1426.00	306.55
Elongation(LW^{-1})	2.10	0.28
Radius variation(µm)	303.37	101.26
Elongation length(L^2/W)µm)	6381.28	2098
Asymmetric major1/(µm)	0.05	0.03
Asymmetric minor1/(µm)	0.05	0.02
Asymmetric ratio	1.02	0.52
Centroid_Hue	4.90	0.29
Centroid_Saturation	2.11	0.64
Centroid_Intensity	11.12	4.13

† The features are defined in Harrell et al. (1991 and 1994)

The data consisted of 75 embryos randomly picked out of 378 embryos from 3 experiments. These 75 embryos of Original set or Analysis set were classified into 23 competent and 52 noncompetent somatic embryos based on their conversion and internal anatomy. The above describes the descriptive statistics of 23 competent somatic embryos. Shaded blocks refers to differences in values between competent and noncompetent class.

Table 4-3 Descriptive statistics of noncompetent class of somatic embryos from the Original or Analysis set.

Feature†	Mean	Std Dev
Area(μm^2)	2170759.37	896031.89
Average radius(µm)	686.04	182.40
Length(L)(µm)	2598.18	639.73
Width(W)(µm)	1188.92	290.62
Elongation(LW^{-1})	2.20	0.40
Radius variation(µm)	277.39	83.04
Elongation length(L^2/W)µm)	5853.24	2185.12
Asymmetric major1/(µm)	0.06	0.04
Asymmetric minor1/(µm)	0.09	0.04
Asymmetric ratio	0.87	0.53
Centroid_Hue	4.74	0.40
Centroid_Saturation	2.34	0.57
Centroid_Intensity	18.13	5.28

† The features are defined in Harrell et al. (1991 and 1994)

The data consisted of 75 embryos randomly picked out of 378 from 3 experiments. These 75 embryos of Original set or Analysis set were classified into 23 competent and 52 noncompetent somatic embryos based on their conversion and internal anatomy. The above describes the descriptive statistics of 23 competent somatic embryos. Shaded blocks refers to differences in values between competent and noncompetent class.

Table 4-4 Morphological feature comparison of competent and noncompetent somatic embryos based on size descriptors using Original or Analysis Set.

Feature†	Level of Significance
AREA	**
AVERAGE RADIUS	**
LENGTH	*
WIDTH	**
ELONGATION	NS
RADUIS VARIATION	NS
ELONGATION LENGTH	NS

Univariate "F" test with DF (1,73)

† The feature are defined in Harrell et al., (1994)
** Significant at 1% level
* Significant at 5% level
NS Not significant

Original or Analysis set consisted of 75 embryos randomly picked out of 378 embryos pooled from 3 experiments. Shaded blocks refers to significant differences between competent and noncompetent classes of somatic embryos

Table 4-5 Morphological feature comparison of competent and noncompetent somatic embryos based on shape descriptors in the Orginal or Analysis Set.

Feature†	Level of Significance
ASYMMETRIC MAJOR	NS
ASYMMETRIC MINOR	*
ASYMMETRIC RATIO	NS
ROUGHNESS	NS
BOX_FILL	NS
CIRCLE_2	NS
SIDE VARIATION	NS
SLOPE VARIATION	NS
SLOPE DIFFERENCE	NS
PARALLEL	NS

Univariate "F" test with DF (1 73)

† The features are defined in Hamill et al. (1991) and in appendix
* Significant at 5% level
NS Not Significant

Orginal or Analysis set consisted of 75 embryos randomly picked out of 378 embryos from 3 experiments. Shaded blocks refers significant differences between competent and noncompetent class

Table 4-6 Morphological feature comparison of competent and noncompetent somatic embryos based on color descriptors of the centroid, radicular and cotyledonary regions of the embryo in the Orginal or Analysis sample

Feature†	Level of Significance
CENT_HUE	NS
CENT_SATURATION	**
CENT_INTENSITY	**
RAD_HUE	NS
RAD_SATURATION	**
RAD_INTENSITY	**
COT_HUE	NS
COT_SATURATION	**
COT_INTENSITY	**

Univariate "F" test with DF (1 73)

† The features are defined in Hamill et al. (1991)
** Significant at 1% level
Significant at 5% level
NS Not significant

Original or Analysis set consisted of 75 embryos randomly picked out of 378 embryos from 3 experiments. Shaded blocks refers significant differences between competent and noncompetent class

Cent refers to centroid region
Rad refers to radicular region
Cot refers to cotyledonary region

This technique is best used when it is necessary to separate known groups (as in the case of our study the competent and noncompetent classes of somatic embryos) and there are quantitative measurements of several variables of individuals under each group (Cruz-Castillo et al. 1994).

CDA (Canonical Discriminant Analysis) generates linear composites of canonical variates (CDF's or Canonical Discriminant Functions) of quantitative variables of individual samples (embryos) and then it separates embryos maximally into two or more groups of individuals while keeping the within group variations small (Hair et al. 1987, Rencher 1992, Cruz Castillo et al. 1994). Since there are only 2 groups (competent and noncompetent), the maximum number of CDF's generated from the sets of variables was only one. In this case, the CDF for a given embryo is proportional to the Fisher Discriminant Function (Afifi and Clark 1990). Based on the mean values of the CDF's of the two groups and the actual value of CDF of each individual embryo, a prediction was made as to which group the embryo fell into. This type of classification is also referred to as posterior probabilities since they exhibit the probability of embryo belonging to a competent or noncompetent group after performing the CDA (Afifi and Clark 1990). A graphical representation of the CDF scores of embryos of size, shape and color variables is presented in Figures 4-2 (a,b), 4-3 (a,b), 4-4(a,b). These pairs of histograms for the competent and incompetent groups revealed that there was a distinct distribution of CDF scores with some degree of overlap.

Once the CDF values were established using the 'Original set' or Analysis sample, the procedure was applied to the Training and Classification set. The performance of the discriminant function in the Original, Training and Classification

Fig 4-2 Distribution of competent and noncompetent classes of somatic embryos based on CDF scores obtained from the size variables

A) Distribution of 23 competent class of somatic embryos based on CDF scores obtained from the size variables

B) Distribution of 52 noncompetent class of somatic embryos based on CDF scores obtained from the size variables

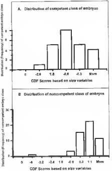

A. Distribution of competent class of embryos
Distribution (Frequency) of competent embryo class
0
2
4
6
8
0
-2.8
1.8
-0.8
-0.3
More
CDF Scores based on size variables
B Distribution of noncompetent class of embryos
Distribution (Frequency) of noncompetent embryo class
0
10
20
30
0
-4
-3.2
-2.4
1.8
-0.8
0.3
1.1
More
CDF Scores based on size variables

Fig 4-3 Distribution of competent and noncompetent classes of somatic embryos based on CDF scores obtained from the shape variables

A) Distribution of 23 competent class of somatic embryos based on CDF scores obtained from the shape variables

B) Distribution of 52 noncompetent class of somatic embryos based on CDF scores obtained from the shape variables

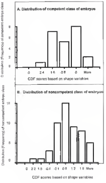
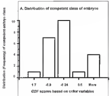
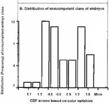

A. Distribution of competent class of embryos
Distribution (Frequency) of competent embryo class
8
6
4
2
0
0
2.4
1.6
-0.8
0
More
CDF scores based on shape variables
B. Distribution of noncompetent class of embryos
Distribution (Frequency) of noncompetent embryo class
15
10
5
0
0
2.2
1.5
-0.8
-0.1
0.8
1.2
1.8
More
CDF scores based on shape variables

Fig 4-4 Distribution of competent and noncompetent classes of somatic embryos based on CDF scores obtained from the color variables

A) Distribution of 23 competent class of somatic embryos based on CDF scores obtained from the color variables

B) Distribution of 52 noncompetent class of somatic embryos based on CDF scores obtained from the color variables


A. Distribution of competent class of embryos


B. Distribution of noncompetent class of embryos

sets are presented in a confusion or classification matrix This matrix compared the classification of competent versus noncompetent embryos

The diagonal entries of the matrix represented the fraction of embryos correctly classified by the analysis The off fraction of the diagonal in the matrix represented incorrect classification The discriminant function in the case of size related features correctly classified embryos as competent 70% 47% and 44% in Original Training and Classification sets respectively (Table 4-7) The noncompetent embryos were classified 85% 73% and 66% in Original Training and Classification sets respectively (Table 4-7)

Based on the shape features competent embryos were classified 78% 48% and 51% and noncompetent embryos were classified 77% 57% and 59% correctly in the Original Training and Classification sets respectively (Table 4-8) For the color related features 78% 55% and 56% of competent and 54% 73% and 57% of noncompetent embryos were correctly classified in the Original Training and Classification sets respectively (Table 4-9) These classification results suggested that misclassification rate was lower in identification of noncompetent classes of somatic embryos using size shape and color features Embryos were correctly classified 70% of time using size and color related features

In order to see any improvement in the classification of competent embryos additional color feature measurements of the cotyledonary and radicular region of embryos were extracted and analyzed No additional improvement resulted with additional color feature extractions (Table 4-10)

Table 4-7 Classification of somatic embryos of sweetpotato into competent and noncompetent embryos based on size descriptors in Original, Training and Classification set.

Original or Analysis set

Class	Competent	Noncompetent	Total
Competent	70%	30%	100%
Noncompetent	15%	85%	100%

Training set

Class	Competent	Noncompetent	Total
Competent	47%	53%	100%
Noncompetent	27%	73%	100%

Classification set

Class	Competent	Noncompetent	Total
Competent	44%	56%	100%
Noncompetent	34%	66%	100%

A total of 378 embryos from three experiments were divided randomly into Original or Analysis set, Training set and Classification set. Original set consists of 75 embryos. Training set and classification set consists of 152 and 151 randomly picked embryos. Discriminant function derived from Original set was applied to the training set. Derived discriminant function from both Original + Training set was applied to the classification set to test the validity. The results of the above are presented in a confusion matrix. Diagonal entries (shaded blocks) refer to correct classification of competent as competent and noncompetent as noncompetent class of somatic embryos.

Table 4-6 Classification of somatic embryos of sweetpotato into competent vs noncompetent based on shape descriptors in Original, Training and Classification set

Original or Analysis set

Class	Competent	Noncompetent	Total
Competent	78%	22%	100%
Noncompetent	23%	77%	100%

Training set

Class	Competent	Noncompetent	Total
Competent	48%	52%	100%
Noncompetent	43%	57%	100%

Classification set

Class	Competent	Noncompetent	Total
Competent	61%	49%	100%
Noncompetent	41%	59%	100%

A total of 378 embryos from three experiments, were divided randomly into Original or **Analysis set**, Training set and Classification set. Original set consists of 75 embryos. Training set and classification set consists of 152 and 151 randomly picked embryos. Discriminant function derived from Original set was applied to the training set. Derived discriminant function from both Original + training set was applied to the classification set to test the validity. The results of the above are presented in a confusion matrix. Diagonal entries (shaded blocks) refer to correct classification of competent as competent and noncompetent as noncompetent class of somatic embryos.

Table 4-6 Classification of somatic embryos of sweetpotato into competent vs noncompetent embryos based on color descriptors in the central region of the embryo in Original, Training and Classification set.

Original or Analysis set

Class	Competent	Noncompetent	Total
Competent	78%	22%	100%
Noncompetent	46%	54%	100%

Training set

Class	Competent	Noncompetent	Total
Competent	55%	45%	100%
Noncompetent	27%	73%	100%

Classification set

Class	Competent	Noncompetent	Total
Competent	56%	44%	100%
Noncompetent	33%	67%	100%

A total of 378 embryos from 3 experiments were placed randomly into Original or Analysis set, Training set and Classification set. Original set consists of 75 embryos. Training set and classification set consists of 152 and 151 randomly picked embryos. Discriminant function derived from Original set was applied to the training set. Derived discriminant function from both Original + training set was applied to the classification set to test the validity. The results of the above are presented in a confusion matrix. Diagonal entries (shaded blocks) refer to correct classification of competent as competent and noncompetent as noncompetent class of somatic embryos

Table 4.10 Classification of somatic embryos of sweetpotato into competent vs noncompetent embryos based on color descriptors of the cotyledonary and radicular region of the embryos in Original, Training and Classification set

Original or Analysis set

Class	Competent	Noncompetent	Total
Competent	74%	26%	100%
Noncompetent	31%	69%	100%

Training set

Class	Competent	Noncompetent	Total
Competent	57%	43%	100%
Noncompetent	32%	68%	100%

Classification set

Class	Competent	Noncompetent	Total
Competent	60%	40%	100%
Noncompetent	47%	53%	100%

A total of 378 embryos from 3 experiments were divided randomly into Original or Analysis set, Training set and Classification set. Original set consists of 76 embryos. Training set and classification set consists of 152 and 154 randomly picked embryos. Discriminant function derived from Original set was applied to the training set. Derived discriminant function from both Original + training set was applied to the classification set to test the validity. The results of the above are presented in a confusion matrix. Diagonal entries (shaded blocks) refer to correct classification of competent as competent and noncompetent as noncompetent class of somatic embryos.

Decision Tree Analysis (CART)

The Decision Tree Analysis also referred as CART-Classification and Regression Tree (Johnson and Wichern 1988) consisted of splitting the data into a smaller number of classes or sects resulting in the grouping of objects that are similar into one class and objects that are not similar into another class (Porter and Anderson 1995 Johnson and Wichern 1988) This splitting of data into subsets is done until no further separation of classes is possible with the split (Porter and Anderson 1995) This is also referred as "Hierarchical Classification" (Gordon 1987) An example of such classification used in biology is the classification system used in taxonomy (Gordon 1987) This technique was used in the present work to split the somatic embryos into competent and noncompetent classes based on morphological measurement related to size shape and color characteristics

The principle involved in splitting the embryos into competent and noncompetent classes is based on a one predictor variable (Porter and Anderson 1995) For example if the width of the embryo was the predictor variable and if it was greater than a particular value (X) then it fell into the competent class if less than X it fell into the noncompetent class The procedure involved in splitting the data into 2 groups at each node or branch was done in such a way that the variability with in group mean was low but variability between group (competent and noncompetent) means was high

The number of branches or nodes of a tree classifier was made by examining and allowing every split among all the predictor variables (Porter and Anderson 1995) For example in our case by examining all the variables under the size shape and color measurements six nodes or branches were optimized for classification of competent and noncompetent embryos under size shape and color variables (Porter and Andersen 1995) The resulting tree was also pruned i e the least important terminal nodes were removed to make a compromise between the tree complexity and tree error (Porter and Anderson 1995) In our present work decision classifier tree analysis was performed based on the above principles described on the size shape and color related measurements of the embryos using the S-plus (Everitt, 1994) the initial input and manipulation data was performed using SAS (SAS Institute Inc. 1990)

The results of the tree analysis of size shape and color related measurement in the Original Training and Classification set are also presented in a confusion matrix (Table 4-11 4-12 4-13) Noncompetent embryos were classified correctly more times than competent embryos based on size, shape and color measurements However the inconsistency of the classification results between the training and classification set questions the applicability of this procedure to the competent and noncompetent embryo classification

Discussion

The significance of these findings presented here is four fold First the percentage of embryos producing both roots and shoots were only half the number of embryos which formed roots Second there were differences in some of the size shape and color variables between competent and noncompetent classes of somatic embryos Third using computer vision and Canonical or Fisher Discriminant Function derived from the morphological feature measurements of anatomically analyzed embryos (see chapter 3) it was possible to correctly classify 50-60% of competent embryos and 70-75% of noncompetent embryos about Fourth of the two classification systems (Canonical Discriminant Analysis vs CART-Classification and Regression Tree Analysis) used to predict the performance of an embryo with regard to competence canonical discriminant analysis was found to be superior

Table 4-11. Classification of somatic embryos of sweetpotato into competent vs noncompetent embryos based on size descriptors in Original, Training and Classification set using CART analysis

Original or Analysis set

Class	Competent	Noncompetent	Total
Competent	**70%**	30%	100%
Noncompetent	6%	**94%**	100%

Training set

Class	Competent	Noncompetent	Total
Competent	**21%**	79%	100%
Noncompetent	16%	**84%**	100%

Classification set

Class	Competent	Noncompetent	Total
Competent	**53%**	47%	100%
Noncompetent	45%	**55%**	100%

A total of 378 embryos from 3 experiments were divided randomly into **Original or Analysis set**, Training set and Classification set. Original set consists of 75 embryos. Training set and classification set consists of 152 and 151 randomly picked embryos. Discriminant function derived from Original set was applied to the training set. Derived decision tree function from both Original + training set was applied to the classification set to test the validity. The results of the above are presented in a confusion matrix. Diagonal entries (shaded blocks) refer to correct classification of competent as competent and noncompetent as noncompetent class of somatic embryos.

Table 4-12 Classification of somatic embryos of sweetpotato into competent vs noncompetent embryos based on based on Shape descriptors in Original Training and Classification set using CART analysis

Original or Analysis set

Class	Competent	Noncompetent	Total
Competent	96%	4%	100%
Noncompetent	19%	81%	100%

Training set

Class	Competent	Noncompetent	Total
Competent	45%	55%	100%
Noncompetent	45%	55%	100%

Classification set

Class	Competent	Noncompetent	Total
Competent	0%	100%	100%
Noncompetent	4%	96%	100%

A total of 378 embryos from 3 experiments were divided randomly into Original or Analysis set, Training set and Classification set. Original set consists of 75 embryos. Training set and classification set consists of 152 and 151 randomly picked embryos. Discriminant function derived from Original set was applied to the training set. Derived decision tree function from both Original + training set was applied to the classification set to test the validity. The results of the above are presented in a confusion matrix. Diagonal entries (shaded blocks) refer to correct classification of competent as competent and noncompetent as noncompetent class of somatic embryos

Table 4-13 Classification of somatic embryos of sweetpotato into competent vs noncompetent embryos based on color descriptors in Original Training and Classification set using CART analysis

Original or Analysis set

Class	Competent	Noncompetent	Total
Competent	**66%**	34%	100%
Noncompetent	19%	**81%**	100%

Training set

Class	Competent	Noncompetent	Total
Competent	**35%**	65%	100%
Noncompetent	15%	**85%**	100%

Classification set

Class	Competent	Noncompetent	Total
Competent	**44%**	56%	100%
Noncompetent	20%	**70%**	100%

A total of 378 embryos from 3 experiments, were divided randomly into Original or Analysis set, Training set and Classification set. Original set consists of 75 embryos. Training set and classification set consists of 152 and 151 randomly picked embryos. Discriminant function derived from Original set was applied to the training set. Derived decision rule function from both Original + training set was applied to the classification set to test the validity. The results of the above are presented in a confusion matrix. Diagonal entries (Shaded Blocks) refer to correct classification of competent as competent and noncompetent as noncompetent class of somatic embryos

The present study (Table 4-1 Fig 4-1) and previous studies (Chee and Cantliffe 1988) showed that most sweetpotato somatic embryos developed roots but not shoots Since the frequency of root formation exceeded shoot formation in all conversion experiments increased regeneration efficiency and whole plant formation thus is highly dependent on the ability to form shoot axis Hence identification of embryos capable of producing shoots was necessary for improved regeneration efficiency and application to synthetic seed technology

Identification of embryos with the greatest potential of conversion into plantlets was the most important requirement in the application of the "synthetic seed" system for clonal mass propagation Heterogeneity of somatic embryos in terms of quality and maturity made it difficult to identify the conversion competent embryos from the noncompetent embryos In such cases a machine vision analysis could be used as tool to select competent somatic embryos based on external morphology characteristics (Harrell et al 1994)

Use of a machine vision could allow consistent selection of somatic embryos that can be difficult to achieve routinely by a biologist over an extended period of time (Harrell et al 1994) Hood and Harrell (1994) developed an embryo sorting system that would maintain the aseptic in vitro condition throughout the harvesting process Using this system Harrell et al (1994) demonstrated automated aseptic harvest of somatic embryos directly from a bioreactor suspension

Hamalainen et al (1992) developed a computer vision system for classification and selection of viable birch embryos Selected embryos would be captured singly using an automated pipette positioned over it and transferred for ex vitro processing (Hamalainen et al 1993) such as a encapsulation or other delivery system (Harrell et a 1994) Sakamoto et al (1992) developed an automated encapsulation system for carrot somatic embryos A vision system was used to detect these encapsulated embryos by quantifying the proportion of green in each gel bead These encapsulated embryos gave 52% conversion in a greenhouse environment. However in order to obtain reliable automated recognition of embryos a specific classification algorithm was needed to avoid much false selection in each species (Hamalainen et al., 1992 1993) Hence the development of a efficient automated classifier and classification system that could identify conversion competent embryos of sweetpotato was neccessary

Using the embryo classifier selection criteria used by an human expert in harvesting the somatic embryos of sweetpotato for conversion studies was mimicked (Harrell et al. 1994) The principle steps involved in the embryo classifier system developed by Harrell et al (1994) consisted of 1) archiving images of embryos 2) deducing algorithms to extract morphological characteristics such as size, shape and color 3) developing a classification structure to categorize the competent and noncompetent embryos used in a conversion study

Geometric parameters of size and shape descriptors are widely used to classify seeds (Shetadel et al 1995) somatic embryos (Cheng and Ling 1994 Grand d'Esnon et al 1988 Hamalainen et al 1992 1993 Harrell et al. 1993 1994 Kurata et al (1991) Molto and Harrell 1992 embryogenic and non embryogenic calli (Grand d'Esnon et al 1988 Harrell et al 1990 Harrell and Cantliffe 1991) Presently color feature extraction is done along with morphological feature extraction using a machine vision system for a number of agricultural products such as variety classification and quality inspection (Liao et al 1994) Newman et al (1989) distinguished wheat varieties using surface color of the grain Paulsen et al (1989) used color image processing to identify fungal damage in soybean Harrell (1991) used such color image processing to distinguish fruits from the background regions in natural tree scenes Harrell et al (1991) also distinguished embryogenic nonembryogenic calli and background in plant callus cultures using color image processing. Thus, morphological cues including color play an important role in identifying competent and incompetent classes of embryos using machine vision.

Statistical analysis (univariate 'F' test - Ott, 1993) showed that there are significant differences in the some of the size, shape and color variables of embryos between competent and noncompetent classes of embryos (Table 4-4, 4-5 and 4-6). Competent somatic embryos had greater mean values for area, width, average radius, length and lower mean values for asymmetric minor and intensity of the centroid (a specific region in the center of the embryo) (Table 4-2 and 4-3). This was not surprising because most of the competent class of embryos are comparatively large, significantly long (Harrell et al., 1993) and were usually well formed with or without some cotyledonary development, creamish white in color compared to non-competent embryos (see chapter 3). Harrell et al. (1994) suggested that an embryo would be symmetric about the major axis and would be asymmetric about the minor axis because of polarity. If both asymmetric major (symmetry about major axis) and symmetric minor (symmetry about the minor axis) were symmetric, then the object (embryo) would resemble circles or ovals (Harrell et al., 1994). Moreover, Harrell et al. (1994) classified embryos as harvestable when the embryo possessed symmetry about the major or principal axis, asymmetry about the minor axis, and hypocotyl region with uniformly sloped sides adjacent to the embryo's center.

A multivariate technique such as Discriminant Analysis can be used in conjuction with computer image analysis to classify seeds (Shatadal et al., 1995) and embryos (Cazzulino et al., 1990). Cazzulino et al. (1990) developed a discriminant function based on 3 feature extraction and classified embryos as non-embryo, globular, heart and torpedo stage embryo. Harrell (1991) and Harrell et al. (1991) applied Bayesian Discriminant Analysis along with color image processing to segment images of fruit and a background region, segment images of embryogenic vs nonembryogenic callus, and plant callus cultures.

Multivariate analysis such as Canonical or Fisher Discriminant Analysis (CDA) and CART technique are also being applied recently in horticultural research. The CDA technique was used in analysis of headspace GC profiles of coffee cultivars (Murato 1993), comparisons of rootstocks of kiwi fruit (Cruz-Castillo et al. 1992) and analysis of supermarket floral marketing (Behe et al. 1992). Porter and Anderson (1995) used the CART technique to identify nutritional and environmental factors affecting sugarcane production.

From the results of both the CDA and CART analyses it was possible to identify the competent and noncompetent embryos using the morphological cues. Comparison of the results from CART with the Fisher Discriminant system (CDA) (Table 4-14) revealed that the Fisher discriminant analysis gave consistent results with the Training and Classification sets. Consistency between training and classification sets was important because they test the validity of the procedure as well as the results (Hair et al. 1987).

Fisher Discriminant Analysis (CDA) exhibited that about 60% of the competent and 60-75% of the noncompetent embryos could be correctly classified as competent and noncompetent, respectively, in terms of their subsequent conversion into plantlets. This resulted in a substantial improvement (about 15%) in regeneration efficiency when compared to the regeneration efficiency realized through manual harvest of embryos which was about 44% (see Table 4-1). In a manual harvest, the embryos are picked by a skilled worker with the use of a stereoscope. This harvesting procedure involved limitations of human vision and quickly becomes tiresome. Over time, this can result in inconsistency in the harvest

Table 4-14 Comparison of performance of Fisher Discriminant Analysis and Decision Tree Analysis on correct classification of Competent and Noncompetent classes of embryos in Training and Classification set

Analysis	Fisher Discriminant Analysis				Decision Tree Analysis			
	Training Set		Classification Set		Training Set		Classification Set	
Feature	C*	NC†	C*	NC†	C*	NC†	C*	NC†
Size	47%	73%	44%	66%	21%	84%	53%	55%
Shape	48%	57%	61%	59%	45%	55%	0%	96%
Color	55%	73%	56%	67%	35%	85%	44%	78%

C* Refers to percentage of Competent embryos correctly classified as Competent
NC† Refers to percentage of Noncompetent embryos correctly classified as Noncompetent

of embryos which are conversion competent Hence the development of an efficient machine vision classifier could identify conversion competent embryos is emulating an expert in harvesting the embryos and yet distinguish slight variation in color that is impossible to achieve accurately with human vision (Harrell et al 1993 and 1994) Use of such a classifier system (Harrell et al 1993 1994) in this study along with information form anatomical studies (chapter3) have resulted in distinguishing the noncompetent from the competent embryos there by improving the chances of regeneration efficiency

Machine vision technology serves as a tool not only for identification of competent embryos but when coupled with a bioreactor system it could permit large scale production of competent embryos that are necessary for commercialization of a synthetic seed system (Cantliffe et al 1993)

Chi et al (1994) showed with image analysis and treatment of TIBA (a polar auxin transport inhibitor) to carrot suspensions that the distribution of morphological features of embryo population were effected More callus clusters twin and secondary embryos were obtained through TIBA treatment (Chi et al 1994) Therefore a continuos monitoring of a developing embryo population through image analysis could allow characterization of environmental effects the effects of plant growth regulators such as auxin aging on long term competent and quality embryo production competent embryo identification and large scale production of somatic embryos

Summary

Sweetpotatoes produce somatic embryos that mature and develop into plantlets in basal nutrient medium The lack of high regeneration efficiency of somatic embryos hinders practical application for transformation technology or synthetic seeding system. Conversion experiments with mature embryos over a 20-day period revealed that 80-90% of the embryos formed roots while 40-50% formed shoots. Using computer vision and Canonical or Fisher discriminant function (CDA) along with conversion results, it was possible to correctly classify competent embryos about 40-50% of the time based on size features, 50-60% based on shape features and 55-60% based on color features. Noncompetent embryos were correctly classified 65-75% based on size, 55-60% based on shape and 70-75% based on color. Of the two classification analysis used namely (CDA) Canonical Discriminant Analysis and CART (Classification And Regression Tree) analysis, CDA was found to be superior as it gave consistent results in classifying competent and noncompetent embryos. This work will provide means to distinguish competent from noncompetent embryos for improved regeneration efficiency.

CHAPTER 5

DIFFERENTIAL AUXIN- AND SUGAR-RESPONSIVE EXPRESSION IN EMBRYOGENIC CALLUS CULTURES OF SWEETPOTATO

(Ipomoea batatas (L.) Lam)

Introduction

Cellular dedifferentiation acquisition of competence and cellular determination are the developmental sequences that precede cellular differentiation during somatic embryogenesis (Christianson 1987 Thorpe 1993) Organized development in these cultures arise through quantitative interactions and balances of diverse growth factors including phytohormones and metabolites (Skoog and Miller 1957)

Auxins play an important role in many growth and differentiation processes including cell enlargement cell division vascular differentiation apical dominance and root formation Both exogenous and endogenous auxin are closely involved in the process of somatic embryogenesis (Michalczuk et al 1992b)

Auxins influence not only those developmental sequences that precede embryo formation but also subsequent morphogenic events in embryo development (Schiavone and Cooke, 1987 Michalczuk et al 1992 a b Zimmermann 1993) Exogenously applied synthetic auxins such as 2 4-D (2 4- Dichlorophenoxy acetic acid) stimulate the accumulation of endogenous IAA (indole acetic acid) (Michalczuk et al 1992b) This maintains the proliferative state of the callus and prevents subsequent embryo formation (Michalczuk et al. 1992b). Removal of callus to a 2 4-D free medium results in the decline of total IAA to levels low enough to set up an internal gradient for initiation and maintenance of polarized growth and subsequent embryo development (Michalczuk et al 1992a)

Auxin induced growth and development involves changes in gene expression (Theologis 1986) Auxin application to plants excised plant organs like soybean hypocotyls (Hagen et al 1984) or tobacco cell cultures (Van der Zaal et al 1987) lead to a rapid increase of many mRNA's and/or genes Several cDNAs corresponding to auxin-induced mRNAs have been isolated These include GH1 GH2 GH3 GH4 SAUR (small auxin up RNAs) (Hagen et al. 1984 Hagen et al 1988 McClure and Guilfoyle 1987) pJCW1 and pJCW2 in soybean (Walker and Key 1982) The soybean GH and SAUR aminoacid sequences are strongly conserved in *Arabidopsis* and pea (Guilfoyle et al 1993) Aux28 and Aux 22 genes corresponding to pJCW1 and pJCW2 cDNAs in soybean are homologous to *Arabidopsis* genes Aux 2-27 and Aux2-11 (Conner et al 1990) When used as probes these cDNA clones can be used as markers for auxin induced expression (Hagen et al 1984 Hagen et al 1988) Stefanov et al. (1990) reported the use of such auxin responsive cDNAs as a tool for screening embryogenic potential and they showed that embryogenic genotypes were more sensitive to 2 4-D treatment than nonembryogenic genotypes

Sucrose the reduced carbohydrate source is reported to be the most effective carbon source for somatic embryogenesis (Ammirato 1983) Sucrose concentration in the media influences both the embryo initiation and differentiation process (Meijer and Brown 1987) In diploid and tetraploid alfalfa genotypes embryo induction was high at sucrose concentrations ranging from 25 to 100mM while embryo differentiation was best at 100mM sucrose (Meijer and Brown 1987). The 100mM range corresponds to 88mM sucrose (3%) used in most standard tissue culture media (Schultheis 1989).

Sucrose utilization for cellular mechanisms in plants is regulated through a group of genes which are modulated in response to changes in sugar levels (Koch et al 1995). These genes include sucrose synthase and invertase which are regulated so as to adjust the capacity for sucrose utilization (Koch et al., 1995).

Sweetpotato somatic embryogenesis requires 2,4-D (10μM) and sucrose (3%) for both callus induction and maintenance. Transfer of embryogenic callus to a 2,4-D free media with 1.6% sucrose leads to embryo production and conversion to plantlets.

Morphogenic potential of sweetpotato cv 'White Star' callus lines decreases with duration in culture. Initially the morphogenic potential of callus lines decreases in terms of conversion of embryos into plantlets and then eventually ceases to produce embryos. One hypothesis for this change is that it may arise from alteration in auxin- and sugar responsive gene expression in callus lines with different morphogenic potential with age. Four auxin responsive cDNA probes namely pJCW1 and pJCW2 (Walker and Key 1982), GH3 and SAUR (Hagen et al 1984; McClure and Guilfoyle 1987) and two sugar responsive gene probes *Sh1* (Sucrose synthase) & *Ivr2* (Invertase) (Koch et al 1992 & 1995) were chosen to test if there is differential auxin- and sugar responsive expression in callus lines differing in morphogenetic potential with age.

Materials and Methods

Basal medium consisted of a semisolid medium with the inorganic salts of Murashige and Skoog (1962) 500μM myo-inositol 5μM thiamine HCl 10μM nicotinic acid 5μM Pyridoxine HCl 3% (w/v) sucrose and 0.7% Phytagel® (Gibco) (Chee and Cantliffe 1988) The pH of the medium was adjusted to 5.8 using 1N sodium hydroxide prior to autoclaving at 121°C for 15 min.

Embryogenic Culture Initiation and Maintenance

Apical and axial shoot tips of *Ipomoea batatas* were used to initiate embryogenic cultures as described by Liu and Cantliffe (1984) Embryogenic callus was gently broken with a sterile glass slide tip and sieved through mesh sized at 710μm and 355μm Callus collected on each sieve was rinsed with 3% (w/v) sucrose solution The resultant 355-710μm fraction was plated onto callus proliferation medium containing modified MS basal medium as described above with 30mM KCl 10μM 2,4-D and 1μM BA (Chee and Cantliffe 1992) Four embryogenic callus lines K592 M892 K1194 K195 were initiated at different times (for example Line K195 was initiated in January 1995) The cultures were maintained in the dark at 30°C in on 5 week culture cycles

Embryo Production , Harvest and Conversion Studies

Somatic embryos arise from callus maintained in auxin containing callus proliferation medium Mature somatic embryos consisting of torpedo and cotyledonary stages were harvested at 7 weeks of callus age in callus proliferation medium Harvested embryos were transferred individually onto embryo conversion medium in small Petri dishes (60x15mm). These embryos were assessed for their root and shoot conversion capacity and callus proliferation in embryos. Embryo conversion medium consisted of basal medium as described above with 1.5% (w/v) sucrose and no growth regulators (Chee and Cantliffe 1992).

Plasmid Preparation and Insert Isolation

Auxin responsive probes

pJCWl and pJCW2 Auxin-inducible cDNA Probes. Plasmids (pBR322) containing the cDNA of two auxin-inducible soybean probes pJCW1 and pJCW2 were obtained from Ainley et al. (1988) University of Georgia, Athens. *E. coli* Cells (HB101) containing the desired plasmid were grown in Luria broth containing tetracycline 20μg/ml at 37°C. Copy number of plasmids were amplified by treatment with chloramphenicol at 150μg/ml overnight (Sambrook et al. 1989). Plasmid DNA was isolated using alkali lysis and purified with the PEG method (Sambrook et al. 1989). cDNA inserts were isolated after restriction digestion with *Pst* I followed by electrophoresis in 0.8% agarose gels (Sambrook et al. 1989). Linearized cDNA inserts (approximate size: pCJW1 - 580 bp and pCJW2 - 720bp) were cut out from the gel and was purified using Prep-A-Gene ® DNA Purification systems, Bio-Rad.

GH3 and SAUR (Auxin-inducible cDNA Probes). Plasmids (pUC19) containing the cDNA of two soybean auxin-inducible probes SAUR and GH3 were obtained from Guilfoyle et al. (1994) University of Missouri, Columbia. *E. coli* cells (DH5α) containing the desired plasmid were grown in Luria broth containing ampicillin 50μg/ml at 37°C (Sambrook et al. 1989). Plasmid DNA was isolated using alkali lysis and purified with PEG method (Sambrook et al., 1989) cDNA inserts were isolated after restriction digestion with *EcoRI* & *Sal* I for GH3 and *Sst* I & *Hind* III for SAUR cDNA clones followed by electrophoresis in 0 8% agarose gels (Sambrook et al 1989) Linearized cDNA inserts (approximate size GH3 1 2 kb and SAUR 250 bp) were cut out from the gel and purified using Prep-A-Gene ® DNA Purification systems Bio-Rad

Sugar responsive cDNA probes Plasmids (pUC 19) containing the cDNA of two sugar-responsive probes *Ivr2* (Invertase gene) and *Sh1* (Sucrose synthase gene) probes from corn were obtained from Koch et al (1992 and 1995), University of Florida Gainesville Plasmid DNA was isolated and purified with the method described above cDNA inserts were isolated after restriction digestion with *Eco* RI for *Ivr2* and *Sst* I for *Sh1* followed by electrophoresis in 0 8% agarose gels (Sambrook et al 1989) Linearized cDNA inserts (approximate size Ivr2 2 2 kb and Sh1 - 4 7 kb) were cut out from the gel and purified as described above

RNA Isolation and Gel Blot Analysis

Embryogenic callus lines K592 M892 K1154 and K195 lines were maintained as described (under Embryogenic Culture Initiation and Maintenance) The embryogenic calli of these lines were harvested at 6 8 and 10 weeks in culture after subculturing Embryo samples were harvested from callus proliferation medium (as described under Embryo Production and Harvest) at 7 weeks of callus age from embryogenic callus line (K195) and were separated as different groups namely 1) Perfect+ Near Perfect 2) Limited/ No Apical Meristem Activity 3) Proliferating+ Disrupted Internal Anatomy Types (a detailed description of these groups is presented in chapter 3) In addition all embryo types from lines K195 (new) and M692 (old) were collected After collection above samples were frozen immediately in liquid N_2

Samples were ground into a fine powder in liquid N_2 and total RNA was extracted (McCarty 1986) RNA was quantified spectrophotometrically (Sambrook et al 1989) Total RNA (10μg) was separated by electrophoresis in 1% agarose gels containing formaldehyde (Thomas 1980) RNA gel was blotted onto a nylon membrane and RNA was fixed to the membrane by UV treatment for 6 minutes (Sambrook et al 1989)

Northern blots were hybridized with linearized cDNA inserts at 47°C for pJCW1 pJCW2 *Ivr2* & *Sh1* probes and at 42°C for GH3 & SAUR probes in a solution containing 7% SDS 250mM Na_2HP04 (pH7 2) and 1% BSA (Church and Gilbert 1984) cDNA inserts were radiolabelled with ^{32}P using random priming kit (BRL)   Blots were washed in a solution containing SDS and Na_2HP04 (pH7 2) (Church and Gilbert 1984) at 49 60°C for the pJCW1 pJCW2 *Ivr2* & *Sh1* probes and at 42°C for the GH3 & SAUR probes   Washed blots were exposed to an intensifying screen of a Phospho Imager (Molecular Dynamics Sunnyvale CA) and also to X ray film with intensifying screens at -80°C

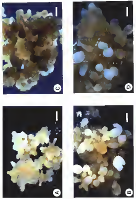
Results

Conversion results

The new embryogenic lines K1194 and K195 gave rise to a greater number of developmentally competent embryos (Figs 5-1 A B C D) compared to old line

Fig 5-1 General physical appearance of embryogenic callus lines of sweetpotato of different age (Bar=1mm)

A Old line K592 (initiated May 1992)
B Old line M892 (initiated August 1992)
C New line K1194 (initiated November 1994)
D New line K195 (initiated January 1995)


A
B
C
D

K592 and M892 The external morphologies of the embryos from the old and new lines were distinctly different even among the torpedo and cotyledonary stage embryos Embryos from the new callus lines (Fig 5-2 C D) were thin and well formed compared to the embryos from the old lines that were short and plump (Fig 5-2 A, B) Most of embryos from old embryogenic lines had the constriction at the shoot apex slightly shifted towards one side (Fig 5-2 A B) These were found to be noncompetent with respect to their capacity for conversion because of lack of apical meristem activity and sparsity of dividing cells at the shoot apical region

The percentage of embryos forming roots in new callus lines was 70-80% while in old callus lines it was about 50-60% (Fig 5-3) Shoot formation in new and old callus lines was 50-60% and 10-20% respectively (Fig 5-4) However the percentage of embryos producing both roots and shoots was only about half those which formed roots regardless of callus age (Fig 5-5) Overall whole plant formation was high in new lines compared to the old lines (Fig 5-5) Plantlets produced from the either age of embryogenic line was normal in appearance (Fig 5-6) Embryo proliferation in (especially at the root pole) which indicates dedifferentiation to callus was higher about 80-90% in the case of old embryogenic lines (K592 & M892) compared to 20-25% in the new embryogenic lines (K195 & K1194) (Fig 5-7) Results of conversion studies indicated that newer callus lines were highly embryogenic and produced competent embryos compared to old lines which produced noncompetent embryos

Fig 5-2. A comparison of general physical features of embryos from callus lines of sweetpotato either recently initiated (new) or repeatedly subcultured over an extended period (old) (Bar=1mm)

A K892 (Old)
B M892 (Old)
C K1194 (New)
D K195 (New)

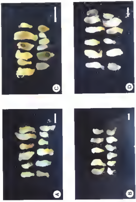

A
B
C
D

Fig 5-3 Root formation in embryos from new and old embryogenic callus lines of sweetpotato

Somatic embryos consisting of torpedo and cotyledonary stages were harvested from different age callus lines at 7 weeks in callus proliferation medium These embryos were transferred individually onto small petri dishes (60x15mm) containing conversion medium Final percentage root formation in embryos were determined after 20 days Data were combined from 3 experiments

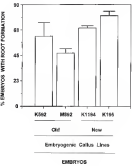

90
68
45
23
0
% EMBRYOS WITH ROOT FORMATION
K592
M892
K1194
K195
Old
New
Embryogenic Callus Lines
EMBRYOS

Fig 5-4 Shoot formation in embryos from new and old embryogenic callus lines of sweetpotato

Somatic embryos consisting of torpedo and cotyledonary stages were harvested from different age callus lines at 7 weeks in callus proliferation medium. These embryos were transferred individually onto small petri dishes (60x15mm) containing conversion medium. Final percentage shoots obtained from embryos were determined after 20 days. Data were combined from 3 experiments.

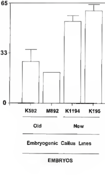

% EMBRYOS WITH SHOOT FORMATION
65
33
0
K592
M892
K1194
K195
Old
New
Embryogenic Callus Lines
EMBRYOS

Fig 5-5 Whole plant formation in embryos from new and old embryogenic callus lines of sweetpotato

Somatic embryos consisting of torpedo and cotyledonary stages were harvested from different age callus lines at 7 weeks in callus proliferation medium. These embryos were transferred individually onto small petri dishes (60x15mm) containing conversion medium. Final percentage whole plants obtained from embryos were determined after 20 days. Data were combined from 3 experiments

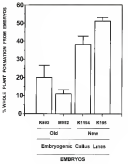

% WHOLE PLANT FORMATION FROM EMBRYOS
60
50
40
30
20
10
0
K592
M982
K1194
K195
Old
New
Embryogenic Callus Lines
EMBRYOS

Fig 5-6 Plant conversion of embryos from new and old embryogenic callus lines of sweetpotato K592 M692(old) and K1194 K195 (new)

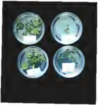
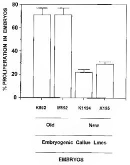


Fig 5-7 Proliferation in embryos from new and old embryogenic callus lines of sweetpotato

Somatic embryos consisting of torpedo and cotyledonary stages were harvested from different age callus lines at 7 weeks in callus proliferation medium. These embryos were transferred individually onto small petri dishes (60x15mm) containing conversion medium. Final percentage of callus proliferation (dedifferentiation) in embryos were determined after 20 days. Data were combined from 3 experiments.


% PROLIFERATION IN EMBRYOS
80
60
40
20
0
K592
M892
K1194
K195
Old
New
Embryogenic Callus Lines
EMBRYOS

Northern Blot Analysis

Auxin responsive cDNA probes

Out of the four auxin-responsive heterologous cDNA probes pJCW1 and pJCW2 hybridized at a higher temperature 48-50°C while GH3 and SAUR cDNA probes hybridized at 42°C The hybridization with GH3 and SAUR cDNA probes under such low stringency conditions resulted in non specific hybridization (results not shown) Hence further analysis were conducted using the more specific probes pJCW1 and pJCW2

Levels of auxin-responsive mRNA hybridizing to pJCW1 and pJCW2 were consistently greater in new (K1194 & K195) compared to the old embryogenic lines (K592 & M892) in calli that had been growing for 6 weeks (Figs 5-8 9) 8 (Figs 5-10 11) and at 10 weeks (Fig 5-12) The relative abundance of pJCW1 and pJCW2 messages as measured by a Phospho-Imager were about half the amount that was expressed in new embryogenic callus at 6 and 8 weeks (Figs 5-8 9 10 11)

To test the existence and identify a threshold for auxin response in embryogenic calli of different ages the 355μm fraction of the (K195) line and (M892) line were exposed to different levels of 2 4-D The 355μm fractions of both lines were grown in suspension cultures without 2 4-D for one day to facilitate partial stripping of auxin After a day in auxin-less suspension medium the 355μm fractions were exposed to different levels (0 1 5 10 20 μM) of 2 4-D for a day Northern blotting and hybridization with both auxin responsive probes (pJCW1 & pJCW2) revealed that the new embryogenic line (K195) and the old embryogenic line (M892) responded in a concentration dependent manner with partial stripping of auxin from calli (Fig 5-13 5-14) However in the new embryogenic line (K195)

Fig 5-8 Evidence for altered expression of an auxin-responsive gene (pJCW1) in new and old embryogenic callus lines of sweetpotato

RNA gel blot analyses were conducted using equal amounts (10µg) of total RNA from embryogenic callus lines K592 M892 (old) and K1194-K195 (new) after 6 weeks in callus proliferation medium Embryogenic cell for RNA extractions were obtained from different aged callus lines of 5 replications (Petri dishes) each Blots were hybridized at 47°C with ^{32}P labeled pJCW1 (auxin-responsive) cDNA probe and rinsed at 48-60°C A Blots exposed to X-ray film for 3 to 4 hours B Blots exposed to Phospho-Imager cassette (Molecular Dynamics Sunnyvale CA) for an hour and abundance of mRNA (pJCW1) quantified Data represent 4 separate experiments and vertical bars denote sweetpotato embryogenic callus lines

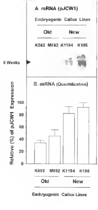

A mRNA (pJCW1)
Embryogenic Callus Lines
Old
New
K592
M892
K1194
K195
6 Weeks
B. mRNA (Quantification)
Relative (%) of pJCW1 Expression
100
80
60
40
20
0
K592
M892
K1194
K195
Old
New
Embryogenic Callus Lines

Fig 6-8 Evidence for altered expression of an auxin-responsive gene (pJCW2) in new and old embryogenic callus lines of sweetpotato

RNA gel blot analyses were conducted using equal amounts (10µg) of total RNA from embryogenic callus lines K592 M882 (old) and K1194 K195 (new) after 6 weeks in callus proliferation medium Embryogenic calli for RNA extractions were obtained from different aged callus lines of 5 replications (Petri dishes) each Blots were hybridized at 47°C with ^{32}P labeled pJCW2 (auxin responsive) cDNA probe and rinsed at 49-50°C A Blots exposed to X ray film for 3 to 4 hours B Blots exposed to Phospho-Imager cassette (Molecular Dynamics Sunnyvale CA) for an hour and abundance of mRNA (pJCW2) quantified Data represent 4 separate experiments and vertical bars denote sweetpotato embryogenic callus lines

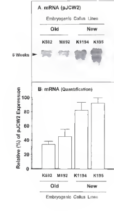

A. mRNA (pJCW2)
Embryogenic Callus Lines
Old
New
K582
M892
K1194
K195
5 Weeks
B. mRNA (Quantification)
Relative (%) of pJCW2 Expression
0
20
40
60
80
100
K582
M892
K1194
K195
Old
New
Embryogenic Callus Lines

Fig 5-10 Evidence for altered expression of an auxin-responsive gene (pJCW1) in new and old embryogenic callus lines of sweetpotato

RNA gel blot analyses were conducted using equal amounts (10μg) of total RNA from embryogenic callus lines K592 M892 (old) and K1194 K196 (new) after 8 weeks in callus proliferation medium Embryogenic calli for RNA extractions were obtained from different aged callus lines of 5 replications (Petri dishes) each Blots were hybridized at 47°C with ^{32}P-labeled pJCW1 (auxin-responsive) cDNA probe and rinsed at 49-50°C A Blots exposed to X ray film for 3 to 4 hours B Blots exposed to Phospho Imager cassette (Molecular Dynamics Sunnyvale CA) for an hour and abundance of mRNA (pJCW1) quantified Data represent 4 separate experiments and vertical bars denote sweetpotato embryogenic callus lines

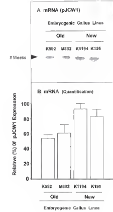

A mRNA (pJCW1)
Embryogenic Callus Lines
Old
New
K592
M892
K1194
K195
8 Weeks
B mRNA (Quantification)
Relative (%) of pJCW1 Expression
100
80
60
40
20
0
K592
M892
K1194
K195
Old
New
Embryogenic Callus Lines

Fig 5-11 Evidence for altered expression of an auxin responsive gene (pJCW2) in new and old embryogenic callus lines of sweetpotato

RNA gel blot analyses were conducted using equal amounts (10μg) of total RNA from embryogenic callus lines K592 M892 (old) and K1134 K195 (new) after 8 weeks in callus proliferation medium Embryogenic calli for RNA extractions were obtained from different aged callus lines of 5 replications (Petri dishes) each Blots were hybridized at 47°C with ^{32}P-labeled pJCW2 (auxin responsive) cDNA probe and rinsed at 49-50°C A Blots exposed to X ray film for 3 to 4 hours B Blots exposed to Phospho-Imager cassette (Molecular Dynamics Sunnyvale CA) for an hour and abundance of mRNA (pJCW2) quantified Data represent 4 separate experiments and vertical bars denote sweetpotato embryogenic callus lines

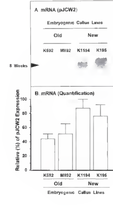

A mRNA (pJCW2)
Embryogenic Callus Lines
Old
New
K592
M892
K1194
K195
8 Weeks
B mRNA (Quantification)
Relative (%) of pJCW2 Expression
100
80
60
40
20
0
K592
M892
K1194
K195
Old
New
Embryogenic Callus Lines

Fig 5-12 Evidence for auxin-responsive gene expression (pJCW1 and pJCW2) in new and old embryogenic callus lines of sweetpotato

RNA gel blot analyses were conducted using equal amounts (10μg) of total RNA from embryogenic callus lines K582 M892 (old) and K1184 K195 (new) after 10 weeks in callus proliferation medium Embryogenic call for RNA extractions were obtained from different aged callus lines of 5 replications (Petri dishes) each A Blots hybridized at 47°C with ^{32}P-labeled pJCW1(auxin-responsive) cDNA probe B Blots hybridized at 47°C with ^{32}P-labeled pJCW2 (auxin-responsive) cDNA probe Blots were rinsed at 48-50°C and exposed to X-ray film for 3 to 4 hours and vertical bars denote sweetpotato embryogenic callus lines

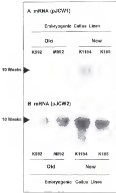

A mRNA (pJCW1)
Embryogenic Callus Lines
Old
New
K592
M892
K1194
K195
10 Weeks
B mRNA (pJCW2)
10 Weeks
K592
M892
K1194
K195
Old
New
Embryogenic Callus Lines

Fig 5-13 Expression of auxin-responsive genes (pJCW1 and pJCW2) in sweetpotato new embryogenic callus line K195

RNA gel blot analyses were conducted with equal amounts (10μg) of total RNA from 355μm fractions of embryogenic callus K195 (new) grown in suspension cultures without 2 4-D for a day and exposed to different levels (0 1 5 10 20 μM) of 2 4-D for an additional day RNA was combined from 2 experiments of 2 replications each Blots were hybridized at 47°C with ^{32}P-labeled pJCW1 and pJCW2 (auxin-responsive) cDNA probes and rinsed at 49-50°C A Blots exposed to X ray film for 3 to 4 hours B Blots exposed to Phospho-imager cassette (Molecular Dynamics Sunnyvale CA) for an hour and abundance of mRNA (pJCW1 and pJCW2) quantified and vertical bars denote sweetpotato embryogenic callus line K195

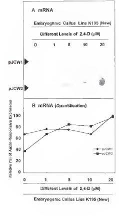
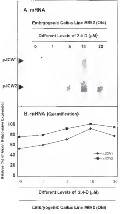

A mRNA
Embryogenic Callus Line K195 (New)
Different Levels of 2,4-D (μM)
0
1
5
10
20
pJCW1
pJCW2
B mRNA (Quantification)
Relative (%) of Auxin-Responsive Expression
100
80
60
40
20
0
pJCW1
pJCW2
0
1
5
10
20
Different Levels of 2,4-D (μM)
Embryogenic Callus Line K195 (New)

Fig. 5-14 Expression of auxin-responsive genes (pJCW1 and pJCW2) in sweetpotato old embryogenic callus line M832.

RNA gel blot analyses were conducted with equal amounts (10µg) of total RNA from 355µm fractions of embryogenic callus M892 (old) grown in suspension cultures without 2,4-D for a day and exposed to different levels (0, 1, 5, 10, 20 µM) of 2,4-D for an additional day. RNA was combined from 2 experiments of 2 replications each. Blots were hybridized at 47°C with ^{32}P-labeled pJCW1 and pJCW2 (auxin-responsive) cDNA probes and rinsed at 49-50°C. A, Blots exposed to X-ray film for 3 to 4 hours. B, Blots exposed to Phospho-imager cassette (Molecular Dynamics, Sunnyvale, CA) for an hour and abundance of mRNA (pJCW1 and pJCW2) quantified and vertical bars denote sweetpotato embryogenic callus line M892.


A. mRNA
Embryogenic Callus Line M882 (Old)
Different Levels of 2,4-D (µM)
0
1
5
10
20
pJCW1
pJCW2
B. mRNA (Quantification)
Relative (%) of Auxin Responsive Expression
100
80
60
40
20
0
pJCW1
pJCW2
0
1
5
10
20
Different Levels of 2,4-D (µM)
Embryogenic Callus Line M882 (Old)

levels of mRNA hybridizing to auxin-responsive probes were maximal only at 20μM 2 4-D (Fig 5-13) compared to an older line (M892) at 10μM (Fig 5-14)

Levels of mRNA hybridizing to auxin-responsive probes varied between samples of embryos classified morphologically (as described earlier in the materials and methods) The relative expression of pJCW1 and PJCW2 message was very high in Limited/No Apical Meristem Activity type embryos when compared to a combination of Perfect + Near Perfect types and Proliferating + Disrupted Internal anatomy types (Fig 5-15 5-16) The response was intermediate for a collection of all embryo types of line K185 and M892 (Fig 5-15 5-16)

Sugar-responsive cDNA probes

The relative abundance of mRNAs hybridizing to the sugar responsive *Sh1* (sucrose synthase) and *Ivr2* (invertase2) gene probes did not vary significantly between the old embryogenic lines (K592 and M892) and the new embryogenic lines (K1184 and K195) either in 8 or 8 week-old callus (Fig 5-17 5-18)

Discussion

The significance of the findings presented here is three fold First newer embryogenic callus lines produced a greater number of developmentally competent embryos that converted readily into plantlets whereas the older embryogenic callus lines produced more noncompetent embryos with poor conversion potential Second expression of auxin-responsive genes was altered in old relative to newer embryogenic lines and differed among embryos with regard to developmental

Fig 5-15 Expression of an auxin-responsive gene (pJCW1) in different embryo types of sweetpotato

RNA gel blot analyses were conducted with equal amounts (10μg) of total RNA from different embryo types 1) Perfect+Near Perfect Type 2) Limited/No Apical Meristem Activity type 3) Proliferating+Disrupted Internal Anatomy type 4) All embryos of line M892 (old) 5) All embryos of line K195 (new) RNA was obtained from at least 100 embryos of each type collected over a 3 to 4 month period and pooled for extraction RNA blot was hybridized at 47°C with ^{32}P-labeled pJCW1 (auxin responsive) cDNA probe and was washed at 49-50°C A Blots exposed to X-ray film for 3 to 4 hours B Blots exposed to Phospho-Imager cassette (Molecular Dynamics Sunnyvale CA) for an hour and abundance of mRNA (pJCW1) quantified and vertical bars denote different sweetpotato somatic embryo types

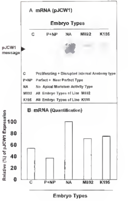

A mRNA (pJCW1)
Embryo Types
C
P+NP
NA
M892
K195
pJCW1 message
C Proliferating + Disrupted Internal Anatomy type
P+NP Perfect + Near Perfect Type
NA No Apical Meristem Activity Type
M892 All Embryo Types of Line M892
K195 All Embryo Types of Line K195
B mRNA (Quantification)
Relative (%) of pJCW1 Expression
100
80
60
40
20
0
C
P+NP
NA
M892
K195
Embryo Types

Fig 5-18 Expression of an auxin-responsive gene (pJCW2) in different embryo types of sweetpotato

RNA gel blot analyses were conducted with equal amounts (10μg) of total RNA from different embryo types 1) Perfect+Near Perfect Type 2) Limited/No Apical Meristem Activity type 3) Proliferating+Disrupted Internal Anatomy type 4) All embryos of line M832 (old) 5) All embryos of line K165 (new) RNA was obtained from at least 100 embryos of each type collected over a 3 to 4 month period and pooled for extraction RNA blot was hybridized at 47°C with ^{32}P-labeled pJCW1 (auxin responsive) cDNA probe and was washed at 48-50°C A Blots exposed to X ray film for 3 to 4 hours B. Blots exposed to Phospho-Imager cassette (Molecular Dynamics Sunnyvale CA) for an hour and abundance of mRNA (pJCW1) quantified and vertical bars denote different sweetpotato somatic embryo types

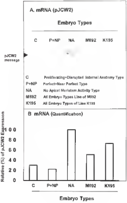

A. mRNA (pJCW2)
Embryo Types
C
P+NP
NA
M892
K195
pJCW2 message
C Proliferating+Disrupted Internal Anatomy Type
P+NP Perfect+Near Perfect Type
NA No Apical Meristem Activity Type
M892 All Embryo Types Line of M892
K195 All Embryo Types of Line K195
B mRNA (Quantification)
Relative (%) of pJCW2 Expression
100
80
60
40
20
0
C
P+NP
NA
M892
K195
Embryo Types

Fig 5-17 Expression of invertase (*Ivr2*) in embryogenic callus lines of sweetpotato of different age

RNA gel blot analyses were conducted with equal amounts (10μg) of total RNA from embryogenic callus lines K592 M892(old) and K1194 K195(new) after 6 and 8 weeks in callus proliferation medium Embryogenic callus for RNA extractions were obtained from different aged callus lines (5 replications (Petri dishes) each) Blots were hybridized at 47°C with ^{32}P-labeled *Ivr2* (sugar responsive) cDNA probe and were rinsed at 49-50°C RNA Blots were exposed to X ray film for 3 to 4 hours

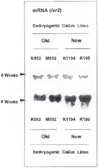
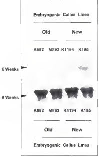

mRNA (*lvr2*)
Embryogenic Callus Lines
Old
New
K592
M892
K1194
K195
6 Weeks
8 Weeks
K592
M892
K1194
K195
Old
New
Embryogenic Callus Lines

Fig 5-19 Expression of sucrose synthase (*Sh1*) in embryogenic callus lines of sweetpotato of different age

RNA gel blot analyses were conducted with equal amounts (10µg) of total RNA from embryogenic callus lines K592 M892(old) and K1134 K195(new) after 6 and 9 weeks in callus proliferation medium. Embryogenic callus for RNA extractions were obtained from different aged callus lines (5 replications (Petri dishes) each). Blots were hybridized at 47°C with ^{32}P labeled *Sh1* (sugar responsive) cDNA probe and were rinsed at 49-50°C. RNA Blots were exposed to X ray film for 3 to 4 hours


mRNA (*Sh1*)
Embryogenic Callus Lines
Old
New
K592
M892
K1194
K195
6 Weeks
8 Weeks
K592
M892
K1194
K195
Old
New
Embryogenic Callus Lines

competence Third sugar responsive genes encoding enzymes of sucrose metabolizing enzymes showed constant expression in both old and new embryogenic lines

Competence of embryogenic callus can be defined as the capacity of the callus to generate embryos which can undergo subsequent conversion into plantlets In sweetpotato newer embryogenic callus lines produced a greater number of competent embryos that converted readily to plantlets whereas the older embryogenic callus lines produced noncompetent embryos with poor conversion potential This reduction of competence or morphogenetic capacity with age may be due to the accumulation of genetic or epigenetic changes or physiological changes due to environmental signals that are lacking such as lack of a particular hormone or inappropriate physical culture conditions (Halperin 1986)

Torrey (1967) and Murashige and Nakano (1967) postulated that genetic changes such as a high level of polyploidy and aneuploidy in cells could be the cause for reduced morphogenetic capacity Smith and Street (1974) found that carrot strains with very little or no embryogenic capacity contained tetraploid, octoploid and aneuploid cells while the embryogenic cells were either diploid or tetraploid giving rise to a diploid or tetraploid embryo However in some studies it was shown that diploid cells may still lose their embryogenic capacity without any chromosomal alterations due to extensive subculturing (Gould 1979 Haddon and Northcote 1976) Smith and Street (1974) found that some carrot cultures lost their embryogenic capacity without any obvious evidence of cytological abnormality Ogihara (1981) suggested that extensive chromosomal rearrangement might occur despite maintenance of a diploid status and this could occur without any obvious effect on the morphology of regenerated organs

The cause of a reduction in embryogenic capacity of sweetpotato callus may relate to genetic changes with age However there is no direct evidence that relates chromosomal abnormalities to loss or reduction in embryogenic capacity or regenerative capacity (Halperin 1986 Ziauddin and Kasha 1990 Henry et al 1996) Moreover genetic imbalances or accumulation of mutations may result in insignificant problems because of the multiplicity of genes in polyploid species (Kao et al 1970) such as sweetpotato which is a hexaploid species In addition Swedlund & Vasil (1985) found that although chromosomal abnormalities were present in callus cultures they were absent from developing somatic embryos These authors suggested that embryogenic cell cultures are less prone to genetic abnormalities due to natural and stringent selection favoring only normal cells to develop during somatic embryo formation.

In the work presented here sweetpotato callus lines over 3 years old remained embryogenic giving rise to many torpedo embryo variants in culture (Figs 5-2 A B) These somatic embryos remained developmentally competent to form roots About 50-60% of the embryos were capable of initiating roots (Fig 5-3) Moreover the somatic embryos from these aged embryogenic lines gave rise to about 10-20% normal looking plantlets (Figs 5-4 5-7) These observations suggest that aged callus cultures were still embryogenic and that significant chromosomal aberrations were apparently not evident in embryogenic callus per se Benzion and Phillips (1988) followed individual pedigree cell lines of embryogenic maize callus cultures and found that distinct aberration starts at a particular stage after the callus induction The complexity of sweetpotato genome (2n=6x=90) does not allow precise cytological studies to be done especially if even slight chromosomal aberration may have occured

Epigenetic or Carry Over' changes in cells involve lack of competence in cells As defined by Halperin (1986) it is the incapability to perceive hormonal or other signals that activate a particular developmental pathway Michalczuk et al (1992b) reported that in order to initiate embryos competence of carrot cells arose from exposure to 2 4-D Such exposure to synthetic auxins such as 2 4-D in turn stimulates the accumulation of high levels of endogenous IAA (indole acetic acid) This action is considered partly responsible for the induction of somatic embryogenesis in carrot cells (Michalczuk et al 1992b) In the present study expression of auxin responsive genes in new embryogenic lines of sweetpotato was more sensitive to auxin induced expression than in old embryogenic lines (Figs 5-9 9 10 11 12) In otherwords the old embryogenic lines which gave rise to noncompetent embryos appeared to be desensitized to auxin (2 4 D) Analysis of auxin responsive gene expression in alfalfa by Stefanov et al (1990) also indicated that the embryogenic genotypes in alfalfa were more sensitive to 2 4-D than the non-embryogenic genotypes

Desensitization to auxin (2 4-D) may involve a feed back mechanism which minimizes the effects of further exposure of embryogenic cells to 2 4-D In *Nicotiana plumbaginifolia* suspension cells Dominov et al (1992) showed there was no further stimulation of mRNA accumulation after the initial peak in levels of a responsive mRNA species with a second addition of 2 4-D They suggested that after the initial response subsided the cells were incapable of subsequent response to the hormonal stimuli Desensitization is an adaptive mechanism in diverse organisms such as bacteria and mammalian systems where organisms loose their capacity to respond to a particular stimulus despite its continued presence or constant intensity (Sibley and Lefkowitz 1985 Benovic 1988)

One of the mechanisms by which desensitization can occur is through down-regulation of receptors (Sibley and Lefkowitz, 1985) A few auxin binding proteins (ABPs) that may function as auxin receptors have been identified and characterized at the molecular level (Venis and Napier 1994) Lo Schiavo et al. (1991) showed that in carrot cell suspensions level of ABPs (auxin binding proteins) in the membranes of the carrot cells was dependent not only on the level of 2 4-D in the medium but also on the physiological status of the cell suspensions They found that young cells contained higher ABPs compared to old cells Filippini et al (1992) used somatic embryogenesis as a model system for studying the modulations in ABPs Using a carrot somatic embryogenesis system they demonstrated that the embryogenic capacity of a given cell line was dependent on its capacity to modulate ABPs They reported that a typical embryogenic line responded to auxin in media by making more ABPs and to its absence by reducing ABP The above studies suggest that embryogenic systems such as the reported here for sweetpotato may loose their capacity to modulate ABPs in response to exogenous auxin concentration This may result in low ABP levels in old embryogenic callus cultures despite the presence of auxin in the medium and hence a desensitization to the auxin (2 4-D) respones

Alternatively desensitization of auxin response may relate to a feed back inhibition as a result of heavy build up of 2 4-D and its metabolites in the old embryogenic callus cells Moore (1989) reported that 2 4-D is a highly persistent auxin and very little of it was destroyed or conjugated because of its resistance to enzymatic degradation and conjugation When suspension cultures of sweetpotato were partially stripped of auxin by incubating in a auxin-less media for a day (Figs 5-13 14) the old embryogenic line (M892) responded to different levels of 2 4-D in a concentration dependent manner This response of old embryogenic lines to various

2,4-D levels was similar to the response of the new embryogenic line (K195) (Figs 5-13 14)

Michalczuk et al (1992a) reported that auxin (IAA) levels in carrot callus were not regulated by auxin feedback mechanisms They found similar growth rates IAA, and 2,4-D levels when comparing embryogenic and nonembryogenic carrot lines grown on 2,4-D supplemented medium However the results presented in this study compared two embryogenic lines and not embryogenic and nonembryogenic lines In young root callus cultures of soybean Davidonis et al. (1978) reported that the level of free 2,4-D increased with the amount of 2,4-D added while the level of 2,4-D conjugates increased very slowly In contrast, the level of free 2,4-D was maintained in mature cultures at near constant levels by increased accumulation of 2,4-D conjugates in response to added 2,4-D Moreover Kuleck and Cohen (1992) reported that the level of auxin conjugates (IAA-aminoacid conjugates) are dramatically reduced during the embryogenic induction process through the action of a developmentally regulated auxin (IAA) conjugate hydrolysis

Together these observations indicated that one possibility for change in embryogenic cultures of sweetpotato with age may be based on a consistently low levels of active auxin (IAA & free 2,4-D) compared to the amount of conjugation of auxin (IAA and 2,4-D) This may result in reduced expression of auxin responsive genes (Figs. 5-8 9 10 11 12) which may not only be due to low levels of active auxin but also to feed back inhibition of auxin responses by build up of conjugated auxins When partially stripped of auxin some of the auxin conjugates may get removed thereby allowing the aged embryogenic lines to show a somewhat concentration dependent response to auxin (Figs 5-13,14) In the same context since the level of free auxin (2,4-D) in mature soybean root callus cultures

(Davidonis et al 1979) and possibly aged embryogenic callus of sweetpotato are maintained at a constant level the ability to modulate ASP may be affected This in turn might affect the desensitization response to auxin (2 4-D)

Auxins influence not only those developmental sequences which precede embryo induction but also subsequent morphogenic events in embryo development (Schiavone and Cooke 1987 Michalczuk et al 1992a Zimmermann 1993) In the presence of auxin the proliferative state of the callus is maintained and subsequent embryo formation is prevented (Michalczuk et al 1992 a b) Zimmermann (1993) suggested that removal of callus to an auxin free medium results in the inactivation of a number of genes allowing the embryo development process to proceed

Additions of auxins such as IAA and 2 4-D at concentrations of 1µM to globular and heart stage embryos prevents subsequent development to the next stage namely heart and torpedo respectively (Schiavone and Cooke 1987) These observations suggest that the development of embryos through successive stages namely globular heart and torpedo requires lower IAA levels than the preceding stage and any addition of external auxin disrupts this gradient In addition proper polar auxin transport is essential for normal embryo development beyond the globular stage (Liu et al 1993 Schiavone and Cooke 1987)

In samples of embryo classes defined by morphological markers for developmental competence (see material and methods) auxin responsive gene expression was altered with respect to their developmental competence Auxin sensitivity was greatest in embryos distinguished in Limited/ No Apical Meristem Activity type embryos and least in Perfect+ Near Perfect type embryos (Figs 5 15 5-16) Michalczuk et al (1992b) reported in carrots that a decline in the total IAA at every stage of carrot embryo development (globular heart, torpedo and cotyledonary stages) allowed further development to the next stage If true for sweetpotato as well the level of total IAA may be very low in Perfect and Near Perfect Type embryos and hence the expression of auxin-responsive genes This low level of IAA activity they may have allowed them to successfully convert to plantlets

The reverse may be occurring in embryos described as Limited/ No Apical Meristem Activity Type where a disrupted polar auxin transport could contribute to auxin accumulation and expression of auxin responsive genes The shoot apices of these embryos are smooth closed and rounded without visible cotyledon development (see chapter 3 for detailed description) Schiavone and Cooke (1987) showed that cotyledon initiation and polarized growth maintenance during the heart stage was dependent on polar auxin transport by using inhibitors of polar auxin transport They also reported that embryos at this stage were sensitive to exogenous auxins Moreover in Indian mustard Liu et al (1993) showed that inhibition of polar auxin transport at the globular stage resulted in mature embryos with fused cylindrical cotyledons similar to the pin1 1 mutant embryo in Arabidopsis (Okada et al. 1991) In carrot embryos LoSchiavo et al (1991) showed that the level of ABP was the lowest in the cotyledons and highest in the hypocotyl The Limited/No Apical Meristem Activity Type embryo were characterized by embryo bodies consisting of a hypocotyl with little or no cotyledon development The high level of expression observed for auxin-responsive expression in these Limited/No Apical Meristem Activity embryo Type could thus be potentially explained in terms of disrupted polar auxin transport and high levels of ABPs in the embryo

Sucrose can enter cellular metabolism through the activity of two enzyme reactions catalyzed by either sucrose synthase or invertase (Huber and Akazawa

1986 Koch et al 1995) Sucrose synthase catalyses the reversible reaction of both formation and cleavage of sucrose but it usually favors the cleavage of sucrose and UDP to fructose and UDP-glucose *in vivo* (Koch et al 1995) Invertases cleave sucrose into glucose and fructose (Koch et al 1995) Two isozymes of sucrose synthase in maize are encoded by *Sh1* and *Sus1* genes respectively (Koch et al 1992) Invertases can be either soluble or insoluble and the former is encoded by a family of four to six genes (Xu et al 1995) Two subfamilies of soluble invertases that were recently distinguished (Koch et al 1995) by hybridization with *Ivr1* or *Ivr2* Expression of these two genes are modulated in response to changes in sugar levels there by facilitating effective utilization of sucrose by importing cells (Koch et al 1995)

Prior to uptake into cells sucrose in the culture medium is first hydrolyzed to hexoses by extracellular cell wall bound invertases (Sanshima et al 1989 Kanabus et al 1986 Felker and Goodwin 1988) Resulting glucose is utilized for respiration while the fructose and UDP-glucose are readily used for sucrose synthesis possibly via sucrose synthase (Sanshima et al 1989 Kanabus et al 1986) In embryogenic callus of sweetpotato the expression of *Ivr2* (Invertase) and *Sh1* (Sucrose synthase) did not show marked variations with callus age (Figs 5-17 5-18) as did the auxin responsive genes This suggested that the capacity for effective utilization of sucrose through modulation of sugar regulated genes was not altered or lost with the reduction in morphogenetic capacity in embryogenic callus of sweetpotato This was consistent with the study in carrot cells where Kanabus et al (1986) reported that the cells grown on sucrose for several years still retained their ability to utilize sucrose

Summary

Both 2 4-D (10µM) and sucrose (3%) are required for induction and maintenance of embryogenic callus in sweetpotato Newly initiated embryogenic callus lines in sweetpotato cv White Star' produce more competent embryos that convert readily into plantlets With age these embryogenic callus lines begin to produce a greater percentage of noncompetent embryos that convert poorly to plants One hypothesis for this reduced conversion might be altered auxin-and sugar responsiveness with age This possibility was addressed by comparing two older embryogenic lines K592 and M892 with two newer lines K1194 and K195 Analyses included conversion studies and Northern blot hybridization with heterologous auxin-inducible gene probes (pJCW1 and pJCW2 Walker and Key 1982 Ainley et al 1988) and sugar responsive gene probes (*Ivr2* and *Sh1* Koch et al 1992 1995) Embryos from new callus lines formed more shoot root and whole plants compared to embryos from old callus lines Northern Analyses revealed that newer callus lines had greater auxin-responsive gene expression while sugar responsive gene expression did not show marked variations between new and old callus lines These results indicated that reduced capacity to generate developmentally competent embryos in callus lines may be related to desensitization of auxin-responsiveness This might be the result of prolonged and repeated exposure to 2 4-D

CHAPTER 6
SUMMARY AND CONCLUSIONS

Over the years somatic embryogenesis has been inducted over 300 species (Bajaj, 1995) in a synthetic media supplemented with auxins and cytokinins (dispensable in some systems). The primary explants from various species and tissues respond to hormonal treatments such as 2,4-D by dedifferentiation of the responding tissue. Through this process a new embryogenic developmental program is triggered in differentiated somatic cells by initiation of cell division and reprogramming of gene expression. However, a complete understanding of mechanisms of the above process and their regulation during somatic embryogenesis is far from being realized. Is it still unclear as to why different species respond differentially to the presence or absence of auxin and why morphogenic capacity varies with different species, cell types (embryogenic vs non embryogenic) and with aging. A starting point for understanding for molecular basis of auxin's involvement in somatic embryogenesis has been provided with identification and involvement of cell cycle protein (p34cdc2 like protein) (John et al., 1993) during auxin-stimulated cell division. Furthermore, identification and purification of various auxin receptors and components of the receptor complex (Lo Schiavo et al., 1995) will further extend our understanding of morphogenetic capacity and behavior of plant cells. Analysis of mutants and transgenic plants could identify key regulatory elements and elements responsible for coordinated gene expression respectively (Dudits et al., 1995). Understanding of physiological and molecular basis of somatic embryogenesis can contribute to improve this system for its practical use as a synthetic seed and for somatic hybridization and transgenic research

Sweetpotatoes produce somatic embryos that mature and develop into plantlets in basal nutrient medium A comparison of external morphology captured via a machine vision system and a study of internal anatomy of the somatic embryos identified five different major morphological variants among torpedo and cotyledonary stage embryos These included 1) Perfect type 2) Near Perfect type 3) Limited/No Meristematic Activity type 4) Disrupted internal Anatomy type 5) Proliferating Type Perfect and Near Perfect types of somatic embryos were categorized as competent while Limited/ No Meristematic activity Disrupted Internal Anatomy Proliferating types were categorized as noncompetent with respect to their ability to convert into plantlets Lack of organized shoot development in somatic embryos of sweetpotato was associated with the following abnormalities 1) lack of an organized apical meristem 2) sparsity of dividing cells in the apical region 3) flattened apical meristem 4) multiple meristemoids and/or diffuse meristematic activity throughout the embryo The morphological fate of most torpedo and cotyledonary embryo variants was mapped which will be beneficial in synthetic seeding and transgenic research and development of sweetpotato

The lack of high regeneration efficiency of somatic embryos hinders practical application for transformation technology or synthetic seeding system Conversion experiments with mature embryos over a 20- day period revealed that 80-90% of the embryos formed roots while 40-50% formed shoots Using computer vision and Canonical or Fisher discriminant function (CDA) along with conversion results it was possible to correctly classify competent embryos about 40-50% of the time based on size features 50-60% based on shape features and 55-60% based on color features. Noncompetent embryos were correctly classified 65-75% based on size, 55-80% based on shape and 70-75% based on color. Of the two classification analysis used namely (CDA) Canonical Discriminant Analysis and CART (Classification And Regression Tree) analysis. CDA was found to be superior as it gave consistent results in classifying competent and noncompetent embryos. This work will provide means to distinguish competent from noncompetent embryos for improved regeneration efficiency.

Both 2,4-D (10μM) and sucrose (3%) are required for induction and maintenance of embryogenic callus in sweetpotato. Newly initiated embryogenic callus lines in sweetpotato cv 'White Star' produce more competent embryos that convert readily into plantlets. With age, these embryogenic callus lines begin to produce a greater percentage of noncompetent embryos that convert poorly to plants. One hypothesis for this reduced conversion might be altered auxin-and sugar-responsiveness with age. This possibility was addressed by comparing two older embryogenic lines K592 and M892 with two newer lines K1194 and K195. Analyses included conversion studies and Northern blot hybridization with heterologous auxin inducible gene probes (pJCW1 and pJCW2, Walker and Key, 1982; Ainley et al., 1988) and sugar responsive gene probes (*Ivr2* and *Sh1*, Koch et al 1992, 1995). Embryos from new callus lines formed more shoot, root and whole plants compared to embryos from old callus lines. Northern Analyses revealed that newer callus lines had greater auxin-responsive gene expression, while sugar responsive gene expression did not show marked variations between new and old callus lines. These results indicated that reduced capacity to generate developmentally competent embryos in callus lines may be related to desensitization of auxin-responsiveness. This might be the result of prolonged and repeated exposure to 2,4-D.

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APPENDIX

Feature Name	Description
AREA	Area contained by the object's contour μm^2
AVE_RADIUS	Average length of 32 radii evenly spaced at 2π/32 rad intervals with origin at object's centroid and terminus on object's perimeter μm
CIR_2	Measure of the conformity of an object's contour to a circle dimensionless
LENGTH	Length of confining rectangle oriented along an object's major axis μm
WIDTH	Width of confining rectangle oriented along an object's major axis μm
BOX_FILL	AREA/(LENGTH * WIDTH) dimensionless
ELONGATION	LENGTH/WIDTH dimensionless
EL_LEN	$LENGTH^2/WIDTH$ μm
RAD_VAR	Squareroot of the sum of the squares of the difference between AVE_RADIUS and the 32 radii μm
ASYM_MAJ	Measure of an object's asymmetry with respect to the major or principal axis 1/μm
ASYM_MIN	Measure of an object's asymmetry with respect to the minor axis 1/μm
ASYM_RATIO	ASYM_MAJ/ASYM_MIN dimensionless
SIDE_VAR	Measure of the nonlinearity or irregularity of an object's contour near the centroid dimensionless
SLOPE_SUM	Measure of the parallelism of an object's sides to major axis dimensionless
SLOPE_DIFF	Measure of the symmetry of slope the object's sides in a region near the centroid dimensionless
PARALLEL	Measure of the conformity in slope of the object's sides in a region near the centroid dimensionless

BIOGRAPHICAL SKETCH

Kala Padmanabhan was born on April 23 in the city of Madras India In 1988 she received her Bachelor of Science in Agriculture from Tamilnadu Agricultural University Coimbatore Tamilnadu India Kala Padmanabhan also received her Master of Science degree in Agricultural Microbiology from the same University in July 1991

She received the prestigious Freedom From Hunger' scholarship form Rotary International to pursue higher education in the field of Agriculture In April 1992 she began her Doctor of Philosophy degree program at the Department of Horticultural Sciences University of Florida, under the supervision of Dr D J Cantliffe Upon completion of her doctoral program Kala plans to continue in plant science and she looks forward to join her husband Ram Kalyanaraman at San Antonio Texas She is the member of Society of In Vitro Biology International Association for Plant Tissue Culture American Society of Plant Physiologists and Alpha Zeta Fraternity

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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August, 1998

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